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INTRODUCTION

The goal of this research was to utilize nonhuman primates to explore a safe, reversible method of menstrual suppression through progesterone antagonist therapy. During a normal menstrual cycle, progesterone (P) secreted in the luteal phase of the cycle primes the endometrium for menstruation. When P levels fall at the end of the cycle menstruation ensues. Progesterone antagonists (antiprogestins, PAs) are compounds that bind to the P receptor and block P action. Continuous blockade of P action during the menstrual cycle by PAs would prevent progestational priming of the endometrium and thus inhibit menstruation. Any unopposed effects of estrogen on the endometrium would also be blocked by another effect of PAs, the endometrial antiproliferative or so-called noncompetitive antiestrogenic effect. The mechanism underlying the "antiestrogenic effect" of PAs was also investigated and new insights were gained on the role of androgens in this effect. Our final report indicates that PA therapy is a reliable, reversible suppressor of menstruation, that the PA can be delivered both systemically, vaginally and through an intrauterine device, and that endometrial androgens may play a role in the endometrial antiproliferative effects of PAs. Once clinically tested and validated in women, PA therapy should provide the military woman with several new options for control of her menstrual periods.

TECHNICAL ISSUES FROM PREVIOUS REPORTS

Response to reviewer's questions from the Year 2 annual report review (June 1999):

Q. Clarify nature and use of Replens™.

A. Replens™ gel is a proprietary product sold over the counter as a human vaginal lubricant. It is an excellent vehicle for PA administration. It is suitable for experimental studies with all steroids for vaginal administration.

Q. Place arrows on figure 1A to denote menstruation onset.

A. All the relevant figures have been redrawn for the manuscript that has been submitted which covers these data. The onset and cessation of menses are now clearly denoted. (See Appendix 1).

Q. Is handling stress a factor influencing hormonal levels, and were these animals acclimated to handling?

A. There was no effect of handling on the hormonal profiles (estradiol, progesterone, LH) in the animals on this study. All the animals in this study were acclimated to handling for several months before the experiments began. All the animals received a food reward at the time of handling including those in the room that were unassigned to an experiment. As a result, there was minimal stress to any of the animals during treatment.

Q. Were the weights of the animals monitored and might the animal's weight reflect the uterine state/size?

A. The animals on this study were weighed monthly as a normal parameter for husbandry. Diets were then modified at monthly intervals to help maintain a constant body weight. There

was a slight trend for all of the animals to gain weight during year 1, this was corrected by diet in year 2. We have found little relationship between animal total size and uterine mass. Any such effect is far less than the changes induced by hormonal treatment.

Response to reviewer's questions from the Year 3 Annual report review (Sept 2000):

Q. The PI needs to review his present SOW to see if a revision is needed with his current focus.

A. Our initial statement of work indicated that we would conduct short term trials (Task 1) in year 1, moderate term trials (Task 2) in year 2, long term trials (Task 3) in year 3 and functional/mechanistic studies (Task 4) in year 4. Because of the excellent data on efficacy and reversibility established through Tasks 1 and 2, we concluded that sufficient information had been obtained to obviate the need for a 180 day trial as originally proposed for Task 3. Of more importance was to evaluate novel modes of PA delivery so we defined Task 3a to evaluate intrauterine device (IUD) delivery and Task 3b to evaluate vaginal gel administration.

Final modifications made to Statement of Work for Task 4.

During our Task 4 research on the mechanisms underlying PA action, we discovered that endometrial androgen receptors were dramatically increased by PA treatment. This was such an unexpected but important finding that we focused our attention almost exclusively on it. Because it was important to validate this finding with more than one PA, we utilized tissues from animals that had previously been treated (under other support) with another PA, RU 486. No animals were treated with RU 486 under this contract.

In addition, we had planned to study various growth factors in the endometrium of PA treated animals. Unfortunately, time did not allow for a full analysis of such factors; efforts to confirm and validate the effects of PAs on endometrial androgen receptors took precedence.

Research Accomplishments:

Task 1-2. Blockade of menses with systemic PA therapy:

The results of these studies were presented at the Annual Meeting of the Society for the Study of Reproduction in 1998 (see list of publications below) and were submitted on September 18, 2000 in a full manuscript for publication in Human Reproduction. A copy of the manuscript with figures is provided in Appendix 1. A summary of the work follows.

The PAs we have studied (ZK 137 316 and ZK 230 211) for menstrual blockade are manufactured by Schering AG, Berlin. During years 1-3, we tested the ability of various systemic doses of ZK 137 316 and ZK 230 211 to inhibit menstruation in cycling rhesus monkeys. We evaluated both short (40 day), intermediate (60 day) and long-term (100 day) treatments. For each PA, a low-dose regimen was established that would block frank menses, and in each case the effects were fully reversible once treatment was stopped. Comparison of these two PAs revealed that ZK 230-211 was 3-5 fold more potent at blocking menses than ZK 137 316. Systemic doses of both PAs that consistently inhibited menstruation in cycling macaques were close to the doses that also inhibited ovulation. Whether ovulation was blocked or not, normal follicular phase levels of estrogen were maintained. This suggests that menstrual blockade with PAs would not result in symptoms of estrogen withdrawal (e.g. bone loss, hot flushes etc.) normally associated with amenorrhea. Of great importance, histological studies revealed no effects of estrogen on endometrial growth; rather, PA induced substantial atrophy of the

endometrium due to the antiproliferative effects of these compounds. In sum these data define a novel method of reversible menstrual suppression by systemic PA therapy in nonhuman primates.

Task 3a. Local Delivery of PAs via Intrauterine Devices.

The studies of systemic administration described above suggested that systemic doses of PA that block menses overlap the doses that are antiovulatory. Although antiovulatory effects may be desirable for contraceptive efficacy or as a means to lower the risk of ovarian cancer, we considered it important to develop methods that would consistently allow ovulation while always blocking menses. Towards this end we hypothesized that an intrauterine device would deliver ZK 230 211 to the endometrium without producing significant systemic effects. Based on measurements of the macaque uterus provided by us, Leiras OY, Finland, a subsidiary of Schering AG, and manufacturer of the Mirena (levonorgestrol) IUD made some PA-containing IUDs designed to release ZK 230 211 at either a high (26-30.2 ug/day) or low (3.3-4.5 ug/day) rate. The IUDs were placed by hysterotomy and tested in ovariectomized-artificially cycled stumptail macaques (*Macaca arctoides*). Both the low and high doses of the ZK-filled IUDs induced menstruation within three days of inserting the IUDs into progestationally primed animals. No major bleeding was detected in the animal treated with the blank IUD (control). These findings indicated that the PA was being effectively released in adequate quantity to locally block the effects of systemic P. After exposure to the IUD for one cycle, uterine biopsies were made, and these revealed that the ZK 230 211 IUDs induced a dramatic, dose dependent inhibition of endometrial development, similar to systemic administration of PA. Moreover, animals with control IUDs menstruated normally at the end of the artificial cycle (when the P implant was removed), but the animals with the ZK 230 211 IUDs did not bleed when the P implant was removed. The failure to bleed when P was withdrawn indicated that the ZK 230 211-releasing IUDs blocked the progestational effects of P so that menses did not follow P withdrawal. Moreover, the effects of estrogen on endometrial proliferation were also blocked, just as we had found after systemic administration of ZK 230 211. No effect of PA was observed in the oviducts of these animals, indicating no systemic effects.

We conclude that PA released by these IUDs was confined to the endometrium and that the antiendometrial effects were due to the action of local, not systemic action. PA-IUD therapy could therefore provide a long-term continuous method of menstrual blockade that allows normal ovulatory cycles. In addition, because of the contraceptive effects of all IUDs, antiprogestin releasing IUDs are likely to provide improved, bleeding-free contraceptive action in women. However, we have not tested the contraceptive efficacy of these devices, as that was not the goal of this work. This study will be presented from platform during the American Society for Reproductive Medicine 2000 meeting (abstract # 0-187). The President of the society has selected this work for announcement at a Press Conference. The abstract is attached as Appendix 2 and the press release is attached as Appendix 3.

Task 3b: Vaginal administration of PAs

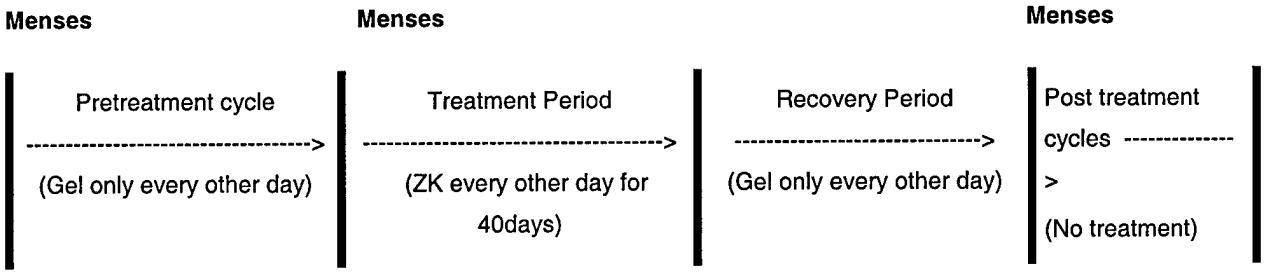
While local IUD administration of PA provides a promising therapy, this technique would require clinical placement of the IUDs for the military woman. We therefore evaluated an alternative method that women could use themselves, namely vaginal administration. Vaginal administration of hormones including P and E_2 is now a clinically accepted mode of delivery that produces significantly higher levels of steroid in the endometrium than in the systemic circulation (Chakmakjian, Z.H. and Zachariah, N.Y., 1987;Tourgeman, D.E., 1999a;Tourgeman, D.E. et al., 1999b). We therefore hypothesized that this "uterine first pass effect" (Bulletti et al., 1997) could

also concentrate PAs within the reproductive tract, block menstruation, and inhibit the endometrium with minimal systemic effects. In year 2 we identified a range of doses of ZK 230 211 that could be delivered every other day to block menses in ovariectomized-artificially cycled macaques. In Year 4 we focused on vaginal delivery of ZK 230 211 in cycling animals.

Methods:

Preliminary dose finding experiments showed that vaginal administration of $\geq$ 10 mg ZK 230 211 every other day would block menstruation in ovariectomized artificially cycled macaques. This dose also inhibited endometrial growth and resulted in atrophic endometria after 28 days of treatment. Subsequently, ovarian intact animals with normal cycle lengths first received vaginal gel vehicle (Replens™, 1 ml) every other day for one cycle (Pretreatment cycle). Beginning on day 0 (menses) of the next cycle the animals received vaginal ZK every other day for 40 days (Treatment period). After ZK treatment the animals were switched back to vehicle (gel) until onset of frank menses (post-treatment period). Two groups were treated: 10mg ZK/dose (n=4), and 15 ZK mg/dose (n=4). A third control group (n=2) received vehicle only for 3 menstrual cycles. This experimental design is shown in diagrammatic form below.

Experimental Design



Results:

The effects of 10 and 15 mg doses of vaginal ZK 230 211 on intermenstrual interval and menses duration in cycling macaques are shown in Table 1. All the animals expressed normal-length pretreatment cycles. Likewise, controls (gel vehicle only) had normal menstrual cycles (29.1 $\pm$ 0.45 days) with normal menstrual periods (3.5 $\pm$ 0.55 days). However treatment with ZK 230 211 extended the intermenstrual interval to ~ 65 days, effectively skipping one normal menses. It appears the vaginal ZK inhibited the endometrium to a very low level even during the recovery period, because the first post-treatment menstruation was minimal (1 day) which suggests a prolonged inhibition of the endometrium. This effect was however fully reversible because the post-treatment menstrual cycles were normal in length (29 $\pm$ 2.1 days) and menstruation was normal in duration (3.1 $\pm$ 0.4 days).

Figure 1 presents hormone profiles for E_2, P and LH levels in the vaginally treated animals. As expected there was no effect of vehicle alone on pretreatment E_2 or P levels. However, vaginal ZK 230 211 at both 10 mg and 15 mg/dose blocked ovulation but permitted normal ovarian follicular phase levels of E_2 (including E_2 surges). In general, treated animals, with blocked ovulation, also failed to develop a significant rise in LH, which suggests a systemic action of vaginally administered PA at the hypothalamic-pituitary level. In one animal treated with 15 mg, however, ZK 230 211 failed to block the LH rise, and ovulation occurred with a normal luteal phase as indicated by levels of ~ 5 ng P /ml serum. In this animal, menses was completely blocked even though the luteal phase was normal and P levels declined at the end of the cycle. Therefore we conclude that even in intact animals, if ovulation occurs and systemic

levels of P are normal, vaginally administered ZK 230 211 can fully block P action on the endometrium and inhibit menses upon P-withdrawal.

In sum, ZK 230 211 can be successfully administered via vaginal gel to inhibit P action in the reproductive tract. Vaginal administration provides an effective mode of ZK delivery that produces antiprogestin levels in the endometrium that block the action of exogenous systemic P and inhibit the proliferative action of systemic E_2. However, ZK 230-211 is such a potent antiprogestin that even when administered vaginally at 10 mg every other day it blocked ovulation in most animals. Further studies exploring lower long-term doses of ZK 230 211 are needed to determine if a vaginal ZK 230 211 regimen can be developed to inhibit the endometrium without systemic actions. Clinical studies are needed to validate this effect in women, but the results in primates indicate this approach would provide an alternative method to inhibit both ovulation and menses.

Results of this work on the effects of vaginally administered ZK 230-211 were presented at the Society for Gynecologic Investigation Annual Meeting (March 2000; abstract # 627, attached as Appendix 4) and completes Task 3 of our Statement of Work. A manuscript on this work is currently in preparation and will be provided once it is complete.

Task 4: Functional/mechanistic studies of PA action.

It is now well-established that chronic treatment of women (Cameron et al., 1996) and nonhuman primates (Slayden et al., 1998) with PAs during the menstrual cycle results in suppression of estrogen-dependent endometrial mitotic activity. The mechanism of this apparent antiestrogenic effect of PAs on cell proliferation is unknown and paradoxical because other well-established effects of estrogen action, including upregulation of estrogen receptor (ER), and P receptor (PR) are hyperelevated during PA treatment (Coates et al., 1996). However, our studies have repeatedly shown that PAs including RU 486, ZK 137 316 and ZK 230 211 also inhibit E_2 stimulated endometrial cell mitotic activity and lead to severe endometrial atrophy (Slayden, O.D. et al., 1993;Slayden, O.D. et al., 1998).

The functional mechanism mediating this "antiestrogenic" effect on cell proliferation is not known. In preliminary studies we showed that infant macaques respond differently to PAs and endometrial atrophy is not induced. This suggests that blockade of E_2-stimulated proliferation requires adult histological, anatomical or physiological relationships within the tissue. In further studies of PA effects in adult animals treated with exogenous steroids we found that PA treatment *dramatically increased endometrial androgen receptor levels.* Because androgens are reported to have dramatic inhibitory effects in the female reproductive system (Futterweit, W. and Deligdisch, L., 1986), we hypothesized that androgens, acting through elevated androgen receptors, might mediate part of the "antiestrogenic" actions of PA.

Currently we have evaluated the effects of various doses of ZK 137 316 (as well as another PA, RU 486) on AR expression in the endometrium of rhesus macaques. This work is nearing completion and a final manuscript will be submitted to JCEM by December 2000. In a very recent, preliminary experiment we analyzed the effect of co-treatment with PAs and the androgen antagonist, flutamide, and found that flutamide partially reversed the antiproliferative effects of PAs on the endometrium. The finding that androgens may play a role in PA action is a novel and exciting one, which we intend to pursue with other support.

Methods:

Nonhuman Primates:

Animal care was provided by the Division of Animal Resources at the Oregon Regional Primate Research Center following the National Institutes of Health guidelines on the care and use of nonhuman primates. Adult rhesus macaques were ovariectomized and then treated sequentially with Silastic capsules containing E_2 for 14 days and then E_2 plus P to induce artificial cycles as previously described (Slayden *et al.,* 1993). At the end of each study shown below the reproductive tract tissues were collected by mid-ventral laparotomy. In each case the uterus was quartered along the longitudinal axis, and cross-sections (2 mm thick) from one uterine quarter were cut with a razor blade and prepared for androgen receptor ICC and in situ hybridization.

PA treatments: The first samples we analyzed for AR expression were from artificially cycled macaques that had been treated in a previous study under separate support (Slayden and Brenner, R. M. 1994). Briefly, fourteen animals had received implants of E_2 for 2 weeks, to create a proliferative endometrium. The animals were then treated for 2 weeks as follows: 1, E_2 only (n = 3), 2, E_2 + P + vehicle (n = 3), 3, E_2 + P + RU 486 (n = 4), and 4, E_2 + RU486 (n = 4). To create these treatments the E_2 implant was left in place, and the animals received either an implant of P after 2 weeks of E, and/or injection with RU 486 (1 mg/kg in ethanol) IM daily.

Another set of 10 ovariectomized macaques were either left untreated for 28 days (n=3) or treated with either E_2 only (n = 4), or E_2 + ZK 137 316 (n = 3). ZK 317-316 (0.1 mg/kg) was administered by IM injection in a nonirritating vehicle (HPE) that consisted of 37.5% Hanks Balanced Salt Solution (Gibco BRL; Grand Island, NY), 37.5% 1,2- propanediol, and 25% ethanol (Aaper, Shelbyville KY).

Bromodeoxyuridine treatment: In our earlier studies we used immunocytochemistry of Ki-67 antigen as a measure of cell proliferation in the endometrium (Slayden, et al., 1994). Bromodeoxyuridine (Br(d)U) is a synthetic thymidine analog. Cells that are actively synthesizing DNA during S-phase of the cell cycle will incorporate Br(d)U into their chromatin, and these cells can be identified immunocytochemically with antibodies directed against Br(d) U. We have now found that in vivo treatment with 100 mg Br(d)U/animal IV in saline effectively labels all cells actively synthesizing DNA in the macaque (Slayden, 2000). This technique does not appear to have untoward effects on the general health of the animals. Therefore, some of the animals treated with PA were also treated with 100 mg Br(d)U/day for 7 days prior to necropsy.

Flutamide treatment. The following preliminary study on the effects of flutamide, a commonly used androgen receptor blocker has been initiated. Beginning at the end of one artificial menstrual cycle macaques (n=9) were treated for 28 days as follows: 1) E_2 implant only (n=4), 2) E_2 + 0.1 mg ZK 137 316 (n=3), 3) E_2 + 0.1 mg ZK 137 316/kg (in HPE) plus 10 mg/kg flutamide (n=1), 4) E_2 + 0.05 mg ZK 230 211/kg (in HPE) plus 10 mg/kg Flutamide (n=1). The flutamide was dissolved in a mixture of 1% DMSO, 20% ethanol and 69% sesame oil. Beginning on day 21 of treatment, each of the animals receiving flutamide were injected daily i.v. with 100 mg of Br(d)U/day. Some of the animals in the E_2 only (n=2), and E_2 plus ZK (n=1) also received Br(d) U.

Laboratory methods

***Histology and Immunocytochemistry (ICC)*:** Samples of fresh tissue for ICC were microwave stabilized and cryosections prepared (Slayden *et al.,* 1995). ICC of AR was done following techniques recently described for estrogen receptor (ER) and P receptor (PR) (Slayden and Brenner, R. M. 1994). Briefly, the microwave-treated sections were lightly fixed in (0.2% picric acid, 2% paraformaldehyde fixative) for 10 min; and then ICC was conducted with monoclonal anti-human AR antibody (F39, Biogenex, San Ramon CA); and antiBr(d)U antibody (ICN Biochemicals). The primary antibodies were reacted with biotinylated anti-mouse IgG and detected with an avidin-biotin peroxidase kit (Vector Laboratories, Burlingame, CA) to give a brown positive stain.

***In situ hybridization (ISH)*:** In situ hybridization was performed using [^{35}S]UTP (DuPont NEN, Boston, MA, USA) -labeled riboprobe from a rhesus monkey-specific 330-base pair AR cDNA (GenBank accession number AF092930). Frozen sections of endometrium mounted on Super Frost Plus slides (Fisher Scientific) were fixed in 4% paraformaldehyde in PBS for 20 min at 4C, rinsed in 2X SSC, then treated with proteinase K (1 mg/ml) at 37°C for 20 min, and acetylated with 0.25% acetic anhydride in 0.1M triethanolamine (pH 8.0) for 10 min. Slides were then rinsed in 2X SSC and air dried. At this point at least one slide per tissue group was treated with RNAse A (20 mg/ml, 0.5M NaCl, 0.01M Tris, 1mM EDTA; pH 8.0) as a negative control. The slides were prehybridized for 1 h at 42C in 10mM DTT, 0.3M NaCl, 20mM Tris pH 8.0, 5mM EDTA, 1X Denhardts solution, 10% dextran sulfate, and 50% formamide. Then sections were incubated at 55C overnight in the same solution containing the appropriate concentration of the sense and antisense probe (5 million cpm/ml). After hybridization the slides were treated with RNAse A containing buffer (20 mg/ml, 0.5M NaCl, 0.01M Tris, 1mM EDTA; pH 8.0) at 37C for 30 min to inactivate nonhybridized probe, and then rinsed in a descending series of SSC (2X SSC, 1X SSC, 0.5X SSC) and then incubated in 0.1X SSC at 65C (high stringency) for 30 min. Sections were dehydrated, vacuum dried, coated with NTB2 autoradiographic emulsion (Eastman Kodak, Rochester, NY, USA), stored at 4C for 2 weeks, and then developed in D-19 (Eastman Kodak).

***Photomicroscopy*:** High power micrographs were captured through Zeiss planapochromatic lenses with the Optronics DEI-750TD CCD camera (Optronics Engineering, Goleta, CA). Digital images were adjusted for sharpness and contrast with Adobe Photoshop (Adobe Systems, Seattle, WA) and photomicrographs were printed with an Epson Stylus Photo 1200 printer.

Results: Androgen receptor expression in the rhesus macaque

Immunocytochemistry. In ovariectomized untreated macaques, specific AR staining was just barely detectable in some of the endometrial stromal cells in the functionalis (Fig. 2i) and basalis zones (not shown). Treatment with E_2 for 28 days resulted in increased specific AR immunostaining in the stromal but not the glandular cells. This E_2-dependent increase in stromal AR was most apparent in the endometrial functionalis (Fig. 2a, j) but also occurred in the basalis zone (Fig. 2e). After sequential E_2 and then E_2 plus P treatment (Fig 2b, f), AR staining intensity was reduced in the stromal cells in the functionalis zone and remained absent from the glandular epithelium. This P mediated reduction was not as clearly evident in the basalis zone where stromal AR was retained (Fig 2f). Specific staining for AR was not detected in the vascular endothelial cells or smooth muscle under any treatment (Fig. 2l).

However, in tissues from animals treated with E_2 + PA, there was a dramatic increase in AR staining not only in the stroma (compare Fig. 2 a-d, e-h and j-k), but also in the glandular epithelium of the functionalis and basalis zones.

In situ hybridization. Figure 3 shows photomicrographs of AR ISH and an analysis of the abundance of silver grains (expressed as a percent of maximum) from the ISH preparations is presented in Figure 4.

In 28-day spayed, untreated macaques, a low level of AR mRNA was detected in the stroma of the functionalis and basalis zones, but glands showed only near-background levels (approximately <5% of the maximum signal). Treatment with E_2 for 28 days significantly ($P \leq 0.05$) increased AR mRNA in the stroma, but not the glands, of both the functionalis and basalis zones (Figure 3 a, d). E_2 plus P treatment reduced the abundance of stromal AR transcript by approximately 40% ($P \leq 0.05$; Fig 3 b and e). However, the highest levels of AR mRNA were evident after combined E_2 + PA treatment, *including a significant increase in glandular AR transcript* ($P<0.05$; Fig 5 d and g). This PA-induced up regulation of AR transcript in the glands was evident in both zones (Fig. 3 c and g). These results confirm the immunocytochemical findings and indicate that PA treatment induces a substantial rise in endometrial AR.

The effects of flutamide. Although preliminary, the data shows that flutamide partially reversed the inhibitory effects of PA on endometrial mass and cell proliferation. The endometrium of the controls treated only with E_2 weighed 700 $\pm$ 61mg /uterus. As expected, cotreatment with ZK 137 316 or ZK 230 211 generated an atrophic endometrium of 120 $\pm$ 30 mg/uterus. However, cotreatment of PAs plus flutamide produced endometria weighing substantially more: 320 mg/uterus than after E_2 plus PA. This represented approximately a 3-fold reversal of the suppressive effects of PA on E_2 action. Additional animals need to be done in order to provide statistical support for this reversal, but the trend for flutamide to reverse PA action was very clear.

Immunohistochemistry showed more nuclei staining for Br(d) U in the glands of the PA plus flutamide animals, than in the absence of flutamide (Figure 5). The number of glandular epithelial cells synthesizing DNA in the E_2 treated controls was 58 $\pm$ 19% (n=2), in the E_2 + PA group, 21% (n=1) and in the E_2 + PA + flutamide group, 44 $\pm$ 8% (n=2). Again, there was a clear trend indicating a reversal by flutamide of the inhibitory effects of PA on DNA synthesis. In the stroma however, there was no effect of flutamide on PA inhibited DNA synthesis. The number of stromal cells synthesizing DNA in the E_2 treated controls, E_2+ PA group, and in the E_2 + PA + flutamide group, was 24 $\pm$ 8%, 6% (n=1), and 2 $\pm$ 0.2%, respectively. This suggests that the novel upregulation of AR by PA in the epithelial compartment may play a key role in the action of PAs on glandular cell proliferation. Although these data are preliminary and need to be extended, the trends indicate that the endometrial antiproliferative effects of PAs are mediated, at least in the glandular compartment, by the androgen receptor. Androgens are known to block the effects of estrogens on endometrial glandular growth, and although androgens circulate in low amounts in females, elevations in androgen receptor levels would increase the effects of androgens in the endometrium. This would go a long way towards explaining how estrogen action is inhibited in the endometrium of PA treated animals.

Tables and Figures

Table 1. Effect of vaginal ZK 230 211 on menstrual cycle length (intermenstrual interval) and menses duration.

	Pretreatment (vehicle only)		Treatment-induced	Post-treatment
Group (n=4)	**Intermenstrual interval**	**Menses Duration**	**Intermenstrual interval**	**Menses Duration**
10 mg	**28.3 ± 1.0**	**3.25 ± 0.29**	**63 ± 1.0[a]**	**1.0***
15 mg	**30.2 ± 2.1**	**3.57 ± 0.60**	**66 ± 6.3[a]**	**1.0***

* All the animals treated with ZK bled for only one day during the post-treatment period.

Figure 1. E_2, P and LH levels in ZK treated animals. There was no effect of vehicle alone on pretreatment E_2 or P levels. Vaginal ZK at 10 mg (panel A; n=4) and 15 mg/dose (panel B; n=3) permitted normal ovarian follicular phase levels of E_2 (including E_2 surges), but blocked ovulation and expression of normal luteal phases. ZK treated animals, with blocked ovulation, also failed to develop a significant rise in LH (panel C), suggesting systemic actions of vaginally administered ZK. M = menses.

Figure 2. Androgen receptor (AR) immunostaining in the rhesus monkey after various treatments. AR was always detected in the nuclei of stromal cells, and only rarely in the glandular epithelium. Treatment with RU 486 and ZK 137 316 resulted in a striking increase in epithelial AR as well as stromal AR. Strong epithelial AR staining was only seen after antiprogestin treatment.

Figure 3. Photomicrographs of ISH of AR mRNA in endometrial sections. AR mRNA was normally expressed in the stromal compartment only. Treatment with PA upregulated AR mRNA in the endometrial glands as well as increasing the stromal signal. Inset (3c) shows negative (RNase pretreatment) control.

Figure 4. Silver grain densities expressed as a percent of maximum.

Figure 5. Br(d)U immunostaining in E_2 alone, E_2 plus ZK 230 211 (E_2+ZK) and E_2 plus ZK 230 211 plus flutamide (E_2 + ZK +FL) treated macaques. Treatment with E_2 + ZK reduced the abundance of cells synthesizing DNA, and co-treatment with flutamide reversed this inhibition.

Key Research Accomplishments

- Continuous short- and long-term systemic treatment with two Schering progesterone antagonists, ZK 317 316 and ZK 230 211, blocked menses in cycling macaques and was fully reversible once treatment stopped. Effective doses blocked ovulation in some of the animals. ZK 230 211 is currently being used by Schering AG in clinical studies and will be available soon for human trials.
- Local administration of ZK 230 211 both by intrauterine device (IUD) and by vaginal administration inhibited the endometrium, but the vaginal route led to systemic effects including inhibition of ovulation.
- Suppression of ovulation has value, both as a contraceptive modality and as a way to lower risk of ovarian cancer.
- IUDs that release ZK 230 211 suppress menstruation effectively and have contraceptive value without blocking ovulation.
- Progesterone antagonist treatment substantially increased the level of endometrial androgen receptor. When PA treatment was combined with the androgen receptor blocker Flutamide, the androgen antagonist substantially blocked the ability of PAs to suppress endometrial growth. The "antiestrogenic" effects of PAs on endometrial proliferation may be mediated by the endometrial androgen receptor.
- Additional clinical trials in women are needed, but the data from primates is compelling that chronic low dose treatment with progesterone antagonists can be useful for the military woman who wishes to control her menstrual cycles.

Reportable Outcomes

- One manuscript has been submitted and three are in preparation
- Four abstracts reporting Tasks 1-4 (Appendix).
- An NIH grant application is being prepared for the February 1 deadline by Dr. Slayden to continue research on the role of androgen receptors in the endometrial antiproliferative effects of PAs.
- Research funds from the Lalor Foundation and the Mellon Foundation were also used to support Dr. Nihar Nayak, who contributed to the IUD work and the in situ hybridization studies reviewed in this report.

Publications 1996-2000

Journal Articles

Slayden OD, Chwalisz K, Brenner RM. Reversible suppression of menstruation with antiprogestins in rhesus macaques: an antiendometrial therapy for uterine bleeding. Hum. Reprod. submitted

Zelinski-Wooten MB, Slayden OD, Chwalisz K, Brenner RM, Stouffer RL. Chronic treatment of female rhesus monkeys with low doses of the antiprogestin ZK 137 316. Establishment of a regimen that permits normal menstrual cyclicity. Hum. Reprod. 13:259-267, 1998.

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prevents pregnancy in rhesus monkeys. Hum. Reprod. 13:2132-2138, 1998.

Slayden OD, Zelinski-Wooten MB, Chwalisz K, Stouffer RL, Brenner RM. Chronic treatment of cycling rhesus monkeys with low doses of the antiprogestin ZK 137 316: Morphometric assessment of the uterus and oviduct. Hum. Reprod. 13:269-277, 1998.

Abstracts

Chwalisz K, Brenner RM, Hess-Stumpp H, Joskowiak D, Elger W. Endometrial effects of progesterone antagonists and novel progesterone receptor modulators (PRMs) in primates. In: Programme for International Symposium on Cell and Molecular Biology of Endometrium in Health and disease (held in Kobe, Japan, October 3-5, 2000).

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Conclusions

A reliable means of menstrual suppression would greatly improve the quality of life for women in occupations requiring strenuous duty such as the military service. Our studies show that chronic, low dose antiprogestin therapy delivered either systemically, vaginally or through an IUD, can provide an alternative method of menses suppression. The dramatic inhibition of endometrial development and bleeding that these compounds induce (Slayden *et al.*, 1998) suggests that they may also serve as a treatment for some menstrual disorders, including dysfunctional uterine bleeding and menorrhagia. Our unexpected discovery that the endometrial androgen receptor is involved in the endometrial antiproliferative effect may explain why estrogen action is opposed during antiprogestin treatment.

So what: The ultimate goal of the USAMRMC program was to support research to improve the health of the military woman. This research has provided new choices for women to suppress their menses, and, because the effective anti-menses doses are near the antiovulatory level, women could use this approach (by dose adjustment) to inhibit ovulation as well. Inhibiting ovulation over multiple years would reduce the risk of ovarian cancer as well as serve as an effective contraceptive method, The safety of the method is enhanced by the discovery that

endometrial androgens appear to play a role in inhibiting estrogen-dependent endometrial growth during antiprogestin therapy. Finally, the potential to suppress menorrhagia and dysfunctional uterine bleeding would go a long way to alleviating these debilitating conditions that plague women at various stages of their reproductive life.

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Appendix

Personnel receiving pay

Publications 1996-2000

News release from ASRM 2000

Figures 1-5

Personnel receiving pay:

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Reversible suppression of menstruation with progesterone antagonists in rhesus macaques.

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Running title: Menses suppression with progesterone antagonists

Keywords: progesterone antagonists/endometrium/menstruation/ovulation/macaque/

Abstract

We evaluated chronic administration of two progesterone (P) antagonists (PAs), ZK 137 316 and ZK 230 211, as a new therapy for controlled, reversible suppression of menstruation. We first established the dose range of these compounds that would either block menses when administered chronically to artificially cycled ovariectomized animals or induce menses when administered acutely at the end of an artificial cycle. Effective doses of ZK 137 316 and ZK 230 211 were then given to naturally cycling animals. The results showed that ZK 137 316 at doses $\geq$ 0.05 mg/kg blocked menstruation in all animals, but 5 out of 9 treated with 0.05 mg/kg and 6 out of 9 treated with 0.1 mg/kg failed to ovulate. In the case of ZK 230 211 all doses that blocked menstruation also blocked ovulation. However, all PA-treated animals maintained normal follicular phase levels of oestradiol and returned to normal menstrual cyclicity within 15-41 days after treatment stopped. Therefore, PA therapy can reversibly suppress menses with specific actions depending on dose and PA type: 1) it can permit ovulation but block endometrial development and suppress menstruation, 2) it can inhibit ovulation so that P levels remain low and amenorrhea results, 3) it can prevent systemic oestrogen deprivation but block unopposed oestrogenic action on the endometrium through its unique antiproliferative effect.

Introduction

As has been recently stressed (Coutinho and Segal, 1999; Thomas and Ellertson, 2000), a reliable means of menstrual suppression would greatly improve the quality of life for women. This is particularly so for occupations requiring strenuous duty and frequent travel; for example, women in the military may especially benefit. The modern woman is accustomed to having control over her reproductive functions, and menstruation is one function that many women would like to control. While oral contraceptives administered without a pill-free interval can be used for this purpose, not all women respond favorably to synthetic oestrogen-progestin combinations, and there are some health conditions (e.g. clotting disorders) that proscribe their use. Progesterone antagonist (PA, antiprogestin) therapy can provide an alternative method of menses suppression. The dramatic inhibition of endometrial growth and development that these compounds induce (Slayden *et al.,* 1998) suggests that they may also serve as a treatment for some menstrual disorders, including dysfunctional uterine bleeding, menorrhagia, and irregular bleeding. These disturbances in the normal cyclic control of menstruation currently represent significant gynecologic diseases affecting up to one third of women of child-bearing age (Fraser *et al.,* 1996; Gould, 1995; Apgar, 1997) and are a leading indication for surgical ablation of the endometrium and hysterectomy (Gould, 1995; Rosenfeld, 1996). Breakthrough bleeding occurs frequently during progestin-only contraceptive therapy and progestin-based hormone replacement as well as during continuous (i.e. without a pill-free interval) oral contraceptive administration (Fraser *et al.,* 1996; Wathen *et al.,* 1995). PAs including mifepristone (RU 486), onapristone (ZK 98 299) and others, as well as newly developed PR modulators (PRMs), either block or modulate P action by binding to the P receptor (PR), and completely (PAs) or partially (PRMs) inhibiting P-dependent gene expression (Spitz *et al.,* 1996; Goodman and Hodgen, 1996; Elger and Chwalisz, 1999). In theory, chronic PA therapy would negate any and all of the effects of progestins that lead to unwanted menstrual bleeding.

Studies evaluating the physiological mechanisms involved in breakthrough and irregular uterine bleeding in women are difficult to conduct. First, it is difficult to predict the onset of breakthrough bleeding episodes in women and therefore, the endocrine and paracrine conditions in the endometrium preceding each event are undefined. Defined and predictable animal models for breakthrough bleeding are also currently unavailable. In contrast, menstruation can be experimentally induced in both women (Critchley *et al.,* 1999) and Old World nonhuman primates (Brenner *et al.,* 1996) by experimental P withdrawal from an oestradiol (E_2) plus P primed endometrium. Menstruation in rhesus macaques therefore provides a hormonally regulated, experimental model that can be used to assess the ability of antiprogestins to suppress uterine bleeding. Data obtained from such experiments are highly relevant to the problem of breakthrough bleeding.

The ability of antiprogestins to inhibit the menstrual cycle was first described in cynomologus monkeys. In that study, high doses of RU 486 (5 mg/day) administered on days 10-12 of the menstrual cycle, delayed the mid-cycle LH surge and lengthened the intermenstrual interval from the normal ~30 days, to 61 days, effectively blocking one menstrual cycle (Collins and Hodgen, 1986). Similar results were reported for women treated with RU 486 during the follicular phase, where RU 486 disrupted follicle maturation and delayed progression of the menstrual cycle (Liu *et al.,* 1987). A single high dose of RU 486 administered to women during the midluteal phase of the cycle also suppressed serum LH pulse amplitude and frequency, was luteolytic and induced menstruation (Garzo *et al.,* 1988). The same workers also reported that corpus luteum function after a single dose of RU 486 could be rescued by exogenous hCG; menstruation was still induced because RU 486 blocked P action in the endometrium.

ZK 137 316 and ZK 230 211 are new-generation PAs with increased potency and reduced antiglucocorticoid activity. ZK 137 316, like RU 486, belongs to type II PAs which in contrast to type I PAs (e.g. onapristone) facilitate binding of PR to DNA and can under certain

circumstances exhibit agonistic activity in vitro and in vivo (Klein-Hitpass *et al.,* 1991; Fuhrmann, unpublished data). ZK 230 211 also promotes a strong PR binding to DNA but, in contrast to type II PR ligands, does not display any PR agonistic activity in vitro or in vivo and can be considered as a pure PA. Based on these properties ZK 230 211 was classified as a type III PA, a novel class of PR ligands (Fuhrmann *et al.,* 1999). It is also more potent than ZK 137 316 in various in vivo models.

In our previously published studies of ZK compounds we investigated the effects of chronic treatment of naturally cycling rhesus macaques with low doses of ZK 137 316. Our goal in that work was to identify an antiprogestin regimen that would allow normal menstrual cyclicity while still inhibiting P-dependent differentiation of the endometrium and be contraceptive. We found that 0.03 mg/kg ZK 137 316 would inhibit endometrial development (Slayden *et al.,* 1998) but still allow menstrual and ovarian cyclicity in half of the animals treated while higher doses inhibited menstrual cyclicity (Zelinski-Wooten *et al.,* 1998a). These lower doses of ZK 137 316 were then shown to be contraceptive (Zelinski-Wooten *et al.,* 1998b), but it was unknown if the effects of ZK 137 316 would be acutely reversible once treatment was stopped. In the current study we evaluated higher doses of these newer PAs to determine whether regimens could be identified that would reliably block menstrual bleeding without also blocking ovarian cyclicity, and whether the effects of such doses would also be reversible. Further, we analyzed the effects of various doses of a Type III PA, namely ZK 230 211, on the same parameters.

Several questions need to be addressed preclinically in macaques to facilitate development of PA-based therapies for menses suppression in women. Can a PA-dose regimen be defined that will reliably inhibit menstruation without also blocking ovarian function in naturally cycling animals? Will PA with different physiological antagonist potency and/or a different molecular mode of action have different effects on ovarian cyclicity and bleeding? Will PA-induced blockade of menstruation be acutely reversible, allowing the blockade of a single menstruation? Will reversibility vary depending on the length of the blockade or the potency of

the antagonist?

To address these questions we first tested the ability of various doses of both ZK 137 316 and ZK 230 211 to antagonize progesterone (P) action on the endometrium in ovariectomized P-primed macaques. Spayed animals were used so that the specific effects on the endometrium could be established, independent of any effects on ovulation. After establishing effective dose ranges for each compound in ovariectomized animals we extended these studies to naturally cycling macaques. The work was done over a three-year period; ZK 230 211 became available for study in the third year.

Materials and Methods

Progesterone antagonists

ZK 137 316 and ZK 230 211 were provided by Schering AG, Berlin, and administered by intramuscular (i.m.) injection in a nonirritating vehicle (HPE) that consisted of 37.5 % Hanks Balanced Salt Solution (Gibco BRL; Grand Island, NY), 37.5% 1,2- propanediol, and 25% ethanol (Aaper, Shelbyville KY). Except where indicated, all hormones and other reagents were purchased from the Sigma Chemical Co (St. Louis MO).

Animal Care

Animal care was provided by the Division of Animal Resources at the Oregon Regional Primate Research Center (ORPRC) following the National Institutes of Health guidelines on the care and use of animals. Chronic treatment of cycling macaques with ZK 137 316 or ZK 230 211 had no obvious effects on general animal health. Experiments were conducted on both intact, naturally cycling rhesus macaques (n=41) and on ovariectomized macaques treated to induce artificial cycles (n=60). Artificial menstrual cycles were created as previously described (Slayden *et al.,* 1993). Briefly, a 3 cm Silastic capsule (0.34 cm i.d.; 0.64 cm o.d.; Dow Corning; Midland MI) filled with crystalline oestradiol (E_2) was first inserted s.c. to induce an artificial follicular phase. After 14 days of E_2 priming, a 6 cm Silastic capsule containing crystalline P was inserted s.c. for an additional 14 days to stimulate an artificial luteal phase.

Removal of the P implant on day 28 completed the cycle and induced menstruation. Serum samples were collected during the artificial cycles to confirm normal levels of E_2 and P.

Menstrual induction in ovariectomized macaques

Menses induction in a P-primed animal is a reliable indicator of antiprogestin action. The threshold doses of ZK 137 316 and ZK 230 211 needed to induce menses were established in E_2 + P primed, artificially cycled macaques as follows. Artificial cycles were induced and beginning on day 28 of an artificial menstrual cycle, the monkeys were injected daily with antiprogestin in HPE vehicle for 7 days while the P implant remained in place. Control animals received vehicle only (n=4). Five doses of ZK 137 316 were tested: 0.01 (n=6), 0.03 (n=6), 0.05 (n=4), 0.1 (n=6), and 0.15 (n=3) mg/kg body weight. Five doses of ZK 230 211 were also tested: 0.005 (n=2), 0.01 (n=4), 0.03 (n=4), 0.05 (n=2), and 0.1 (n=2) mg/kg. Vaginal swabs were performed daily for 9 days and the ability of the various doses of antiprogestin to induce frank, overt menses (blood detectable on the external genitalia and the cage floor) or minute bleeding detectable only by vaginal swab was recorded.

Menstrual blockade of P-withdrawal menses by chronic administration of PAs in artificially cycled, ovariectomized macaques.

The minimum dose of chronically administered ZK 137 316 needed to block menses after P-withdrawal in artificially-cycled, E_2 + P primed macaques, was determined as follows. The animals were injected with ZK 137 316 daily in HPE during a complete cycle (E_2 for 14 days and then E_2+P for 14 days). Control animals received vehicle only during the artificial cycle (n=4). Four doses of ZK 137 316 were tested: 0.01, 0.03, 0.05, and 0.1 mg/kg body weight (n=3 each). At the end of the cycle, the P implants were removed, and injections of ZK 137 316 were continued for 7 more days. Vaginal swabs were performed daily for 9 days after P implant removal and the incidence of frank menses or minute bleeding was recorded.

Histological effects of ZK 230 211 in ovariectomized hormone-treated macaques

Previously we described the histological changes induced by low doses of ZK 137 316

on endometrial growth and development in naturally cycling macaques treated for 5 menstrual cycles (Slayden *et al.,* 1998). Here we tested the effects of various doses of ZK 230 211 on endometrial differentiation after treatment for one cycle. Five groups of ovariectomized macaques were treated as described above to create artificial menstrual cycles. Beginning on day 1 of the second cycle the animals were treated as shown in tabular form below. Animals treated with E_2 alone were included to provide a baseline measure of the degree of E_2-dependent proliferation.

Treatment	Description
E_2 alone (n=4)	E_2 for 28 days
E_2 + P (n=4)	E_2 for 14 days then E_2 plus P for 14 days (1 artificial cycle)
E_2 + P + 0.005 mg ZK (n=3)	1 artificial cycle plus 0.005 mg/kg ZK 230 211 daily
E_2 + P + 0.016 mg ZK (n=3)	1 artificial cycle plus 0.016 mg/kg ZK 230 211 daily
E_2 + P + 0.032 mg ZK (n=3)	1 artificial cycle plus 0.032 mg/kg ZK 230 211 daily

At the end of treatment, reproductive tracts were collected and the uterus was separated from the cervix and oviducts. The uterus was quartered along the longitudinal axis, and cross-sections (2 mm thick) from two uterine quarters were cut freehand with a razor blade and prepared for immunocytochemistry (ICC) and morphological study. Endometrial and myometrial weights were obtained from the remaining two quarters after the endometrium was separated from the myometrium with fine scissors. The oviducts were dissected free from fat and connective tissue, and weighed. Samples of fimbriae and ampulla were prepared for morphological study. Details of tissue handling were as follows.

Histology and immunocytochemistry

Tissue samples for morphological study were fixed in 2% glutaraldehyde and 3% paraformaldehyde, embedded in glycol methacrylate (GMA), sectioned (uterus, 2 µm; oviduct

1.5 µm) and stained with Gill's Hematoxylin (Slayden *et al.*,1998). Samples of fresh tissue for immunocytochemistry (ICC) were microwave stabilized (Slayden *et al.*,1995) in an Amana Radarrange Touchmatic microwave oven (Amana, Iowa) for 7 sec in 0.5 ml Hank's Balanced Salt Solution (Gibco), then chilled on ice in 10% sucrose dissolved in 0.1 M phosphate buffered saline (PBS; Sigma), mounted in Tissue Tek II OCT (Miles Inc., Elkhart, IN) and frozen in liquid propane. Cryostat sections (5 µm) were thaw-mounted on Superfrost Plus (Fisher Scientific Pittsburgh, PA) slides, placed on ice at 5C, and microwaved for 2 sec. ICC of oestrogen receptor alpha (ERα), P receptor (PR) and Ki-67 was done as recently described (Slayden *et al.*, 1998). Briefly, the microwave-treated sections were lightly fixed (0.2% picric acid, 2% paraformaldehyde in PBS) for 10 min; and the ICC was conducted with monoclonal anti-ERα (1D-5; Biogenex, San Ramon CA), anti-PR (JZB-39; provided by Geoffrey Greene, University of Chicago) or anti-Ki-67 antigen (Dako Corp. Carpinteria, CA). In each case primary antibody was reacted with either biotinylated anti-mouse IgG (for 1D-5 and anti Ki-67) or anti-rat IgG (for JZB-39) second antibody and detected with an avidin-biotin peroxidase kit (Vector Laboratories, Burlingame, CA).

Photomicrography

Low power photographs were made with an Olympus OM-system 38 mm macro lens on Technical Pan film (Eastman Kodak, Rochester, NY). Negatives were digitized with a Polaroid Sprintscan 35 Plus film scanner. High power micrographs were captured through Zeiss planapochromatic lenses with the Optronics DEI-750TD CCD camera (Optronics Engineering, Goleta, CA). Digital images were adjusted for sharpness and contrast with Adobe Photoshop (Adobe Systems, Seattle, WA) and photomicrographs were printed with an Epson Stylus Photo 1200 printer.

Morphometrics

Abundance of mitotic cells, Ki-67 positive cells, and apoptotic cells in the endometrium were determined by a trained observer who used an ocular micrometer grid to define

microscope fields and counted between 1200-5000 cells per animal with the aid of a mechanical tabulator. Mitotic index represented the number of mitoses per 1000 epithelial cells. Endometrial stromal cell density values (stromal compaction) were determined with the Optimas 3.0 image analysis software package. For this analysis, 10 nonoverlapping fields of endometrial stroma (30,000 um^2 each) in the upper functionalis of each specimen were analyzed. The number of stromal cell nuclei per unit area (standardized to 10,000 um^2) provided an index which reflected the degree to which the endometrial stroma became expanded (more edematous) or compacted (less edematous). Apoptotic cell counts were done in high quality, glycol methacrylate sections; the cytological markers of apoptosis can easily be distinguished in such sections. Percent apoptosis was based on a total count of 5000 glandular epithelial cells.

Multiprotocol assessment of menstrual blockade with PAs in naturally cycling macaques

Untreated, adult macaques were first monitored for 2 complete menstrual cycles to document normal cycle lengths for each animal prior to treatment. Three independent protocols were conducted in naturally cycling animals and these protocols are depicted graphically in Figure 1 and described below.

Protocol 1: Short term (40 day) treatment with ZK 137 316: Beginning on the day after the onset of menstruation (day 2), adult macaques with normal menstrual cycles were injected daily with ZK 137 316 in HPE for 40 days (see Fig. 1). Three groups were treated: HPE vehicle only (control; n=4), 0.05 mg/kg (n=9), and 0.1 mg/kg (n=9). Daily blood samples and vaginal swabs (to detect bleeding) were collected during the treatment period until the animals displayed a menstruation of longer than 2 days. After menstruating, the monkeys were allowed to rest one menstrual cycle, and then daily blood samples were again collected for one menstrual cycle. All blood samples were analyzed for concentrations of E_2 and P. Lengths of the menstrual cycles, intermenses interval, and serum hormone levels were compared between treatment groups.

Protocol 2: Long term (100 day) treatment with ZK 137 316 : Starting on day 2 of menses, animals with normal menstrual cycles were injected daily for 100 days with ZK 137 316 in HPE.

Three groups (n=4 each) were treated: 1) vehicle only (control), 2) 0.05 mg ZK 137 316 /kg, and 3) 0.1 mg/kg body weight. Daily vaginal swabs were collected during the entire treatment period. Daily blood samples were collected for analysis of E_2 and P during the last 30 days of treatment and continued until the monkeys menstruated (2 days). The monkeys were further monitored for menses during the first post-treatment cycle.

Protocol 3: Intermediate (60 day) treatment with ZK 230 211: During protocol 1 and 2 we showed that HPE vehicle injection had no effect on hormone levels, menstrual cycles or endometrial bleeding patterns. Therefore, in protocol 3, macaques were first injected i.m. daily with HPE alone for one control, pretreatment cycle. Then beginning on the day after menses in the next cycle, the animals received ZK 230 211 in HPE (i.m.) daily for 60 days. Three doses of ZK 230 211 (at steps of approximately 3 fold) were compared: 1) 0.005 mg/kg, 2) 0.016 mg/kg and 3) 0.05 mg/kg body weight (n=5 each). Daily vaginal swabs (to detect minute bleeding) and daily blood samples were collected during the pretreatment cycle, the treatment period (60 days) and the recovery period, until the monkeys menstruated for longer than 2 days. The monkeys were further monitored for menstruation by vaginal swab without blood collection during the first post-treatment cycle. Blood samples were assayed for serum concentrations of E_2 and P, and once these levels were known, samples that flanked the surge (or highest value) of E_2 were assayed for bioactive LH.

Hormone assays

All blood samples were analyzed for concentrations of E_2 and P by routine radioimmunoassay and LH determinations were made by bioassay (Zelinski-Wooten *et al.,* 1998a). All assays were performed by the ORPRC Hormone Assay Core.

Statistical analysis

Quantitative data were statistically compared by analysis of variance (ANOVA). Significant differences among means were determined by Fisher's protected least significant difference test (Petersen, 1985). These data included lengths of the pre-treatment menstrual cycles,

treatment-induced intermenses interval, length of the recovery period (time to return to menses) after the last injection, post-treatment menstrual cycle length, tissue weights, percent fimbrial ciliation, mitotic index, percent Ki-67 positive epithelial cells, apoptotic index and degree of stromal compaction.

Results

Ovariectomized animals

Menstrual induction: The ability of short term administration of various doses of ZK 137 316 and ZK 230 211 to induce menses in E_2 plus P-primed, ovariectomized animals is shown in Table 1. Serum levels of E_2 and P produced by Silastic implants in these animals were within the normal range for the luteal phase (mean ± SE; 89 ± 27 pg E_2/ml and 4.39 ± 0.48 ng P/ml).

Treatment with 0.01 mg/kg ZK 137 316 daily for 6 days did not induce either menses or swab-detectable bleeding. Injection of 0.03 mg/kg ZK 137 316 daily induced uterine bleeding detectable only by vaginal swab. Increasing the treatment dose to 0.1 mg/kg induced frank menses (detectable on the external genitalia and/or cage floor) in half the animals, and 0.15 mg/kg consistently induced full menses in all animals. With ZK 230 211, 0.005 mg/kg had no effect on menses, but 0.01 mg/kg induced frank menses in 2 out of 3 animals tested, and doses of $\geq$ 0.03 consistently induced frank menses. These data suggest that ZK 230 211 was approximately 3 fold more potent than ZK 137 316 in inducing uterine bleeding.

Menstrual blockade of P withdrawal bleeding with ZK 137 316: Table 2 shows the effects of continuous administration of various doses of ZK 137 316 on menstruation induced by P withdrawal in artificially cycled, ovariectomized animals. As expected, all the HPE injected control animals menstruated when P was withdrawn at the end of the artificial cycle. Treatment with 0.01 mg/kg ZK 137 316 daily during the artificial cycle did not prevent P withdrawal menses. At the 0.03 mg/kg dose, frank bleeding was detected in one out of three animals; the other two animals showed only minute bleeding. Increasing the treatment dose to $\geq$ 0.05 mg/kg blocked all P withdrawal bleeding, indicating that this dose provided a full blockade of P action

in the endometrium.

Together, the results on menstrual induction and blockade define the effective range of antiprogestin action in the endometrium of ovariectomized, artificially cycled rhesus macaques. Based on these results we selected two doses of ZK 137 316 (0.05 and 0.1 mg/kg) and three doses of ZK 230 211 (0.005, 0.016 and 0.05 mg/kg) for further study of menstrual bleeding patterns in naturally cycling monkeys.

Histological effects of ZK 230 211: Animals treated with E_2 + P alone displayed an hypertrophied, progestational (secretory) endometrium (Figure 2a) with sacculated glands and expanded stroma in both the functionalis and basalis zones (Figures 2a and e), and well developed spiral arteries (Figure 2i). In the basalis zone, the glandular epithelium was tall columnar and mitotically active. Under this hormonal condition, the epithelium of the oviductal fimbriae was deciliated and cuboidal (Fig. 2m), indicative of the normal suppressive action of P on the oviduct (Brenner and Slayden, 1994).

Treatment with ZK 230 211 at 0.005 mg/kg had little or no effect on endometrial differentiation. In contrast, doses of 0.016 and 0.032 mg/kg inhibited progestational differentiation of the endometrium (Fig. 2c-d) and blocked the suppressive effects of P on E_2 action in the oviduct (Fig. 2o-p). Compared to vehicle injected controls, the higher doses of ZK 230 211 resulted in an overall thinning of the endometrium (compare Figure 2a-d), which was maximal in the 0.016 mg/kg and 0.032 mg/kg groups. This effect was associated with significantly increased stromal compaction (Table 3) and a decrease in number and sacculation of endometrial glands (compare Fig. 2 e-f with g-h). These higher doses of ZK 230 211 also caused hyalinization of the walls of the spiral arteries (Fig. 2k-l) especially the adventitial layer, indicative of arterial degeneration. At the highest dose, ZK 230 211 also produced some venous dilation (Figure 2 d) and the dilated veins also showed increased hyalinization of the perivenous stroma (not shown).

ZK 230 211 at 0.005 mg/kg had no effect on oviductal differentiation or secretion, (Figure 2n), but the 0.016 and 0.032 mg/kg doses resulted in oviducts that were fully differentiated (Figure 2c and d) as indicated by a substantial increase in percent ciliation (Table 3, $P<0.05$); these data indicate that the two higher doses of ZK 230 211 were able to completely block the suppressive effects of P on E_2-dependent oviductal differentiation.

Immunocytochemical effects of ZK 230 211: The effect of each dose of ZK 230 211 on endometrial ER, PR and Ki-67 is presented in Figure 3. In E_2 + P-treated control animals, ER, PR and Ki-67 staining in the functionalis was generally low because of the suppressive action of P (Brenner *et al.*, 1990; Okulicz *et al.*, 1993; Hild-Petito *et al.*, 1992). Treatment with ZK 230 211 at 0.005 mg/kg had no effect but 0.016 and 0.032 mg/kg blocked P suppression and increased ER (compare Fig. 3 a-d) PR (Fig. 3 e-h) and Ki-67(Fig. 3 i-l) staining in glands and stroma.

Morphometric indices affected by ZK 230 211: Table 3 shows that endometrial thickness and mass were significantly suppressed by ZK 230 211 at doses of 0.016 and 0.032 mg/kg. At these doses, thickness and mass were not only below the amount stimulated by E_2 +P treatment , but also below the level induced by E_2 alone. Similarly, ZK 230 211 at doses of 0.016 and 0.032 mg/kg suppressed the mitotic index in the functionalis well below that stimulated by E_2 alone. These data indicate that ZK 230 211 can induce an endometrial antiproliferative effect, that is, a blockade of E_2-dependent mitosis. However, there were no differences in the effects of treatment with 28 days of E_2 versus ZK 230 211 on the Ki-67 index. (Table 3).

In the basalis zone of the macaque, mitosis is P-dependent, not E_2-dependent (Brenner and Slayden, 1994); consequently there was considerable Ki-67 and mitotic activity in this zone in the animals treated with E_2 + P alone. ZK 230 211 treatment with both the 0.016 and 0.032 mg/kg doses dramatically suppressed the Ki-67 and mitotic indices in the basalis.

In the E_2 + P treated animals, the apoptotic index was significantly lower than that seen after E_2 treatment because P treatment tends to suppress endometrial apoptosis. ZK 230 211 at doses $\geq$ 0.016 mg/kg, blocked the action of P on apoptosis and raised the level to that seen under E_2 alone (Table 3).

Naturally cycling macaques

Suppression of menses with ZK 137 316 and ZK 230 211: Table 4 presents the menstrual cycle lengths of monkeys injected with vehicle or various doses of ZK 137 316 and ZK 230 211. Because there was no difference in cycle length in vehicle control-treated animals, cycle length data in these monkeys from protocol 1 and 2 have been pooled. All of the animals in the study exhibited normal pre-treatment cycle lengths. Effects of treatment with ZK 137 316 and ZK 230 211 for different periods are described below; a summary is shown in Table 5.

Short-term (40 day) treatment with ZK 137 316: Injection with ZK 137 316 (0.05 and 0.1 mg/kg) blocked frank menstruation in all of the monkeys during the 40 day treatment period (Tables 4,5). In the 0.05 mg group, 4 monkeys showed minute bleeding detectable only by vaginal swab on days 27-30 of the treatment period. In the 0.1 mg group 2 monkeys also showed minute, swab-positive bleeding on days 27-28 of the treatment period. Normal menstrual cyclicity began anew 15-23 days after treatment ceased and all animals maintained normal menstrual cyclicity thereafter. There was no significant effect of ZK 137 316 treatment on post-treatment cycle lengths.

Figure 4 presents E_2 and P levels in control macaques treated with HPE alone for 40 days. There was no effect of vehicle on the normal cyclic pattern of E_2 and P during the menstrual cycle. Figure 5 shows E_2 and P levels in macaques treated with ZK 137 316 for 40 days (shaded area). During the treatment period, monkeys in the control group expressed normal menstrual cycle patterns of E_2 and P. In the 0.05 mg/kg group, ovulation was unaffected in 4 animals and suppressed in 5 animals, but in all animals, non-surge E_2 levels were normal

(~30-100 pg/ml). Two of the animals with normal E_2 surges failed to develop a normal luteal phase. Similar results were seen in the 0.1 mg group, with 6 of the 9 monkeys not showing an E_2 surge and 7 of the 9 monkeys not developing a normal luteal phase. In both ZK 137 316-treated groups, the monkeys that showed normal luteal phases maintained the luteal phase for 9-14 days and then P declined to baseline by day 27-30 of the treatment period. Frank menses was blocked in these animals despite normal levels of P and normal decline in P at the end of the luteal phase; only minute bleeding, detectable by swab, was observed at the time of expected mense. This minute bleeding was not observed in the ZK 137 316-treated animals that failed to develop a luteal phase. Figure 6 shows E_2 and P levels during the second post-treatment menstrual cycle. During this period, animals in all the groups expressed normal patterns of E_2 and P and normal menses indicating that there were no residual effects of ZK 316 on the ovary and endometrium .

Long-term (100 day) suppression of menses with ZK 137 316: Compared to vehicle injected control animals, injection with ZK 137 316 blocked frank menstruation and significantly extended the intermenstrual interval in all of the monkeys during the treatment period. The mean (± SE) treatment intermenstrual intervals in the control, 0.05 and 0.1 groups were 28.1 ± 0.83, 131.1 ±1 0.1 and 134 ± 8.7 days, respectively ($P<0.001$). The time to recovery of menstrual cyclicity after treatment stopped was approximately 31-35 days. In this group, blood samples were collected only for the last 30 days of treatment. During this 30 day period the monkeys in the control group expressed normal menstrual cycle patterns of E_2 and P (Figure 7). All of the monkeys in the 0.05 mg/kg group, (n=4) had normal follicular phase levels of E_2 (including an E_2 surge) and normal luteal phase levels of P. In the period 70-80 days, when a clear luteal phase was evident, ZK 137 316 suppressed all menstruation, including minute bleeding, despite the normal decline in luteal phase P at the end of the cycle.

No minute bleeding was detected in the 0.05 mg group at approximately 78 days of treatment when P levels declined, or at other times prior to 70 days when P decline was

anticipated (blood samples not taken). In the 0.1 mg/kg group, all of the monkeys failed to develop either a normal E_2 surge or normal luteal phase levels of P during the last 30 days of treatment. Despite blockade of ovulation in this group, all of the monkeys showed normal non-surge levels of E_2 (~30-100 pg/ml).

When treatment ended (at 100 days) most of the animals in the 0.05 mg/kg group quickly developed an E_2 surge and ovulated (Figure 7B) within 5 days In the 0.1 mg/kg group ovulation was delayed by ~15-17 days. Both groups expressed a normal luteal phase after ovulation in the recovery period, and all of the animals menstruated as P declined indicating that the menstrual blockade was quickly reversible once treatment stopped. Post-treatment cycle lengths were normal in all of the groups (29.8 ±3.0 days).

The combined results of the short and long term studies with ZK 137 316 suggest that treatment with 0.05 mg/kg, long or short term, is near but generally below the threshold dose that blocks ovulation and suppresses luteal phase P. However, 0.1 mg/kg did alter ovarian function and prolonged treatment blocked both E_2 surges, ovulation and luteal phase P, though baseline E_2 levels remained well within physiological range. There was no obvious residual effect of antiprogestin and effects appeared completely reversible, as post-treatment cycles were normal in all respects.

Intermediate term (60 day) suppression of menses with ZK 230 211: Effects of 60 day ZK 230 211 treatment on menstrual cycle length are presented in Table 4 and hormone profiles are depicted in Figure 8. As expected, all of the groups exhibited normal length pretreatment menstrual cycles (27.6 ± 2.1 days), with normal E_2 surges and normal luteal phase levels of P. During the treatment period, injection with the lowest dose of ZK 230 211 (0.005 mg/kg) had no effect on menstrual cyclicity, and all the animals menstruated normally at 27.2 ± 1.0 day intervals. In contrast, injection with 0.016 mg and 0.05 mg ZK 230 211/kg blocked frank menstruation and significantly extended the intermenstrual interval in all of the naturally cycling

animals to approximately 100 days ($P<0.01$; Table 2). In these two groups, the recovery period, from the time treatment stopped to the onset of the first post-treatment menses , was 34 ± 6 days. Both 0.016 mg and 0.05 mg doses also slightly increased the length of the post-treatment menstrual cycle to approximately 50 days ($P<0.05$); menstrual cycles thereafter were of normal length (27.5 +2.1 days).

In this protocol we bled the animals daily throughout the entire study period until the first menstruation occurred after the recovery period. Animals in the 0.005 mg/kg groups expressed normal menstrual cycle patterns of E_2 and P throughout the treatment, consistent with a sub-threshold dose. Macaques treated with 0.016 mg/kg daily displayed follicular phase E_2 surges of normal amplitude (Figure 9*).* However, normal luteal phase levels of P did not develop in animals treated with 0.016 mg/kg, suggesting a blockade of ovulation. Monkeys treated with 0.05 mg/kg failed to develop either a normal E_2 surge or normal luteal phase levels of P. Despite blockade of E_2 surges in this group, all of the monkeys showed normal non-surge levels of E_2 (~30-100 pg/ml) throughout the treatment period. Approximately 10 days after treatment ended, both groups demonstrated a rise in serum P, and a normal length luteal phase. Menses occurred following P decline at the end of this luteal phase (Fig. 8).

In order to determine if inhibition of ovarian function by ZK 230 211 involved suppression of LH, serum samples flanking the highest levels of E_2 in each group were re-assayed for LH by bioassay (bLH, Figure 9). Samples from animals during the pretreatment period, the recovery period, and those treated with 0.005 mg ZK 230 211 all showed a rise of bLH associated with peak levels of E_2. However, those animals treated with ZK 230 211 at 0.016 and 0.05 mg/kg showed no increase in bLH, indicating that these doses were high enough to suppress pituitary LH secretion. This effect on the pituitary was fully reversible, as the post-treatment cycles showed normal LH surges.

Discussion

In order to establish appropriate dose ranges for PA effects in the endometrium of

nonhuman primates, we first conducted dosage trials with both ZK137 316 and ZK 230 211 in artificially cycled ovariectomized macaques. The data showed that doses of antiprogestin that failed to induce menses also failed to block menses when administered throughout the artificial cycle, and that menses-inducing doses blocked P-withdrawal bleeding when administered continuously throughout the artificial cycle. When the work was extended to naturally cycling animals, the effective doses were close to the ranges established in ovariectomized animals. Moreover, the data established, for the first time, that ZK 230 211 had direct suppressive effects on the primate endometrium at a dose ~3-5 fold lower than ZK 137 316.

ZK 137 316: The 40 and 100 day studies .

The two treatment periods, one for 40 and one for 100 days, were designed to mimic those cases where women might want to block either one or several menstrual periods. During the 40 day study (Fig 5, Table 5) ovulation was blocked in 5/9 animals at 0.05 mg/kg and 6/9 animals at 0.1 mg/kg. In the animals whose ovulation was blocked, E_2 levels were within physiological ranges, but there was no P secretion, no P withdrawal, and consequently no menstruation. In those animals in which ovulation occurred, the luteal phase length, the serum levels of P and E_2, and the time of decline of P were normal, but there was no frank menstrual bleeding. Undoubtedly this was due to the combined antagonistic effects of ZK 137 316 on both E_2 dependent growth (the endometrial antiproliferative effect, (Wolf *et al.,* 1989; Slayden and Brenner, 1994; Slayden *et al.,* 1998) and P-dependent endometrial progestational development (Brenner and Slayden, 1994).

In the 40 day study, the ovulatory animals showed minute bleeding, detectable only by vaginal swab, around the time of P decline. These positive vaginal swabs detected bleeding that was far less in quantity than the so-called spotting or breakthrough bleeding which occurs in women on continuous progestin treatment (e.g. Norplant, or Depo Provera). Women would not notice what we have defined as minute bleeding. Moreover, in the 100 day study, the animals were completely amenorrheic with neither frank nor minute bleeding. The reason for the

differences between the two studies, which were done one year apart, is not clear. However, if all the data from the current study on ZK 137 316 are combined, the results suggest that doses of ZK 137 316 that allow ovulation may sometimes, but not always, allow minute bleeding (detectable only by swab) at the time of P decline, and that these doses are near the threshold for blocking ovulation. For instance in the 0.05 mg/kg group, there was clear evidence of a luteal phase during days 70-80 of treatment, but not during days 93-100 (see Fig. 7). Therefore, over times greater than 100 days, ovarian cyclicity may be blocked by this dose.

The ability of ZK 137 316 to inhibit endometrial differentiation may have additional benefits to women undergoing chronic therapy. Recently, Zelinski-Wooten et al., (Zelinski-Wooten *et al.,* 1998a; Zelinski-Wooten *et al.,* 1998b) showed that a lower, chronically administered dose of ZK 137 316 (0.03 mg/kg) prevented pregnancy but allowed normal ovarian function and menstruation. The higher doses we used should be even more effective for contraception. The rapid return to normal ovarian and menstrual cyclicity after both the 40 and 100 day treatments indicate that women could easily restore normal cyclicity, and presumably fertility, within a short time after antiprogestin-induced menstrual blockade.

ZK 230 211: Histological and morphometric effects.

ZK 230 211, a new, highly potent type III PA dramatically suppressed the endometrium in E_2 plus P-primed animals at 0.016 mg/kg, a dose ~62 fold lower than the effective dose of RU 486 under similar conditions (Slayden and Brenner, 1994) and at least 2 fold lower than the previously reported dose of ZK 137 316 that blocked the endometrium in naturally cycling monkeys (Slayden *et al.,* 1998). There were specific histological effects of overall shrinkage, stromal compaction, glandular atrophy and hyalinization of the spiral artery walls. In addition, the suppressive effects of P on endometrial ER and PR were blocked, and ZK 230 211 also blocked the antagonistic effects of P on E_2 action in the oviduct. These effects were similar to those produced by long-term (5 months) administration of ZK 137 316 in intact, naturally cycling animals (Slayden *et al.,*1998). However, this is the first report to show that ZK 230 211 can

induce these effects directly on the endometrium and oviduct within 28 days in ovariectomized, artificially cycled, E_2 + P treated macaques.

The data in Table 3 indicate that ZK 230 211 suppressed endometrial mass, endometrial thickness and the functionalis mitotic index significantly below the level in animals treated with E_2 alone. In other studies, we have treated some ovariectomized rhesus macaques with E_2 + ZK 230 211 alone, in the absence of P, and found a clear, dose-dependent and dramatic suppression of E_2-dependent mitosis (unpublished data, in preparation). Because ZK 230 211 treatment elevated both ER and Ki-67 levels, the findings suggest that E_2 acts through ER (directly or indirectly) to stimulate cells to produce Ki-67 and enter the cell cycle, but some unknown aspect of ZK 230 211 action blocks the cells from entering mitosis. The exact mechanism underlying this endometrial antiproliferative effect still remains to be discovered.

In addition, ZK 230 211 elevated apoptotic counts, which are very low in the E_2 + P treated control animals, to the level seen during E_2 treatment alone (Table 3). Because ZK 230 211 treatment suppressed the mitotic rate but raised the apoptotic index, cell death would greatly outpace cell birth in the functionalis zone, and over time this difference would contribute to the decrease in endometrial cell mass and thickness induced by these doses. In the basalis, which grows under P influence, the blockade of P action by ZK 230 211 would block the growth of this zone as well. Suppression of growth of both zones contributes to the overall shrinkage of the endometrium. Clearly, moderate doses of ZK 230 211 can block the effects of both E_2 and P on endometrial growth and development.

ZK 230 211: The 60 day treatment of cycling animals.

ZK 230 211 failed to suppress either menstruation or ovulation at 0.005mg/kg, but completely suppressed both ovulation and menstruation at the ~3 fold higher dose of 0.016 mg/kg. The suppression of the LH surge induced by this and the 3-fold higher dose of 0.05

mg/kg clearly indicates that this compound had central effects resulting in suppression of LH secretion. In a previous publication, we noted that the antiovulatory effects of ZK 137 316 were also accompanied by suppression of LH surges, implicating the hypothalamic-pituitary axis as the site of the antiovulatory action for both these antiprogestins (Zelinski-Wooten *et al.,* 1998a).

Conclusions

These data indicate that the antiovulatory and antiendometrial effects of ZK 137 316, a Type II PA, were somewhat dissociated, that is, menstruation could be inhibited in approximately half the animals without inhibiting ovulation. On the other hand, ZK 230 211, a Type III PA, blocked ovulation at all doses that blocked menstruation. Moreover, other data indicate that ZK 230 211 is a pure PA, while ZK 137 316 has some agonist action (Chwalisz *et al.,* 2000). These results suggest that pure PAs are most suitable for therapeutic approaches which require a consistent inhibition of ovulation, whereas compounds exhibiting some agonistic activity, such as ZK 137 316, and particularly the PRMs, appear to be more endometrium-selective.

Of great importance, however, circulating E_2 levels were never suppressed below normal, follicular phase levels by either PA at any dose. Therefore, if women used these compounds to block menses, and if ovulation were also blocked, there would be no associated symptoms of oestrogen deprivation, such as hot flushes, vaginal atrophy or bone density loss. Moreover, the antiovulatory effects of PAs would be accompanied by ovarian secretion of naturally occurring oestrogens, an important difference from the current mode of menstrual inhibition, namely ovulation blockade obtained from the use of combined synthetic oestrogens and synthetic progestins in a continuous contraceptive tablet regimen. Synthetic progestins are usually administered to prevent unopposed actions of synthetic oestrogens on the endometrium, but PA therapy, through its antiproliferative effects, can obviate the need for such treatment.

Chronic low dose PA therapy is, therefore, a powerful new method to reversibly suppress menstrual bleeding, as it can act in several ways depending on dose and PA type: 1)

it can allow ovulation, but block the effects of both E_2 and P on endometrial growth and development, suppress menstruation upon P withdrawal and induce a state of amenorrhea, 2) it can inhibit ovulation so that P levels remain low and no P withdrawal occurs, which also causes amenorrhea, 3) it can allow normal follicular phase levels of E_2 so that no systemic oestrogen deprivation occurs, and 4) it can block any unopposed oestrogenic effects in the endometrium through its unique endometrial antiproliferative effect. In sum, PA therapy represents a new modality to provide women with more control over their menstrual periods. The potential also exists for suppression of various sorts of abnormal uterine bleeding and for enhanced contraceptive efficacy. Long term studies in women are needed to validate these possibilities, but the data from nonhuman primates are compelling.

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Figure Legends

Figure 1. Experimental designs for assessment of menstrual blockade with PAs in naturally cycling macaques.

Figure 2. Photomicrographs of GMA-embedded, haematoxylin-stained sections of endometrium and oviduct. Full thickness photos of endometrium in the upper row (a-d) are shown at the same magnification (Bar indicates 1 mm). The dark line was drawn to indicate the endometrial-myometrial border. Glands (e-h) and arteries (i-l) were photographed at 250X, and oviduct (m-p) was photographed at 400 X original magnification. After sequential E_2+P treatment the endometrium displayed an hypertrophied-progestational state (a), with sacculated glands (b) and hypertrophied spiral arteries (l). ZK 230 211 at 0.005 mg/kg had only minimal effects (b), but at 0.016 and 0.032 mg/kg, ZK 230 211 induced a dose-dependent thinning of the endometrium , decreased progestational differentiation, and increased stromal compaction (compare a-d and e-h). These higher doses of ZK 230 211 induced degenerative accumulation

of matrix around the spiral arteries (k and l arrows). At the highest dose, ZK 230 211 also produced some venous dilation (d; arrow). In the oviduct E_2 + P resulted in a deciliated (m) and nonsecretory state that was unaffected by 0.005 mg/kg ZK 230 211. ZK 230 211 at 0.016 and 0.032 mg/kg blocked P action and resulted in oviductal differentiation into a fully ciliated state (m and p). C= ciliated cells, Se = secretory cells, Gl=glands, S=stroma, Endo=endometrium, Myo=myometrium.

Figure 3. Color photomicrographs of endometrium immunostained (brown) for ERα (a-d), PR (e-h) and Ki-67 (I-l). In E_2 + P-treated animals, ER (a) and PR (b) staining and the abundance of Ki-67 positive cells was minimal. ZK 230 211 at 0.016 and 0.032 mg/kg resulted in increased intensity of ER and PR staining and an increase in the abundance of Ki-67 positive cells (arrow). ER and PR micrographs were photographed at 250X and Ki-67 at 100X original magnification. S=stroma, Gl = glands.

Figure 4. Mean (±SE) E_2 and P levels in control macaques treated with HPE alone for 40 days. Days indicates time from the onset of menses during the menstrual cycle. The area under the curve of P levels is dark shaded in this and subsequent graphs. Vertical arrow and M indicates approximate time of menstruation.

Figure 5. Mean (± SE) E_2 and P levels in macaques treated with 0.05 mg/kg ZK 137 316 (A) and 0.1 mg/kg ZK 137 316 (B) for 40 days. Days indicates time from the onset of menses during the treatment menstrual cycle. Hormone profiles are shown for animals where ovulation was unaffected on the left, and suppressed on the right. Recovery period represents the time span from the end of treatment to the onset of menses. The duration of treatment is indicated with light shading in this and subsequent graphs.

Figure 6. E_2 and P levels (mean ± SE) during the second post-treatment menstrual cycle after a 40 day treatment with ZK 137 316. All of the animals expressed normal patterns of E_2 and P and normal menses, which indicates no residual effects of ZK 137 316.

Figure 7. E_2 and P levels (mean ± SE) during the last 30 days of treatment in macaques treated

with vehicle (A), 0.05 mg/kg ZK 137 316 (B), and 0.1 mg/kg ZK 137 316 (C) for 100 days. Days represent time from the beginning of treatment.

Figure 8. Mean (±SE) E_2 and P levels in animals treated with 0.005 mg (A), 0.016 mg (B), and 0.05 mg/kg ZK 230 211 (C) for 60 days.

Figure 9. Levels (mean ± SE) of bioactive LH (bLH) in animals treated with ZK 230 211. Samples were first analyzed for E_2. In cases where a clear E_2 surge occurred, as in panels A and B, samples flanking the surge were reassayed for bLH. When no surge was evident, as in panel C, samples flanking the highest value of E_2 were reassayed for bLH.

TABLES

Table 1. Menstrual induction: bleeding patterns induced by various doses of ZK 316 and ZK 211 in progestationally-primed macaques. U= undetectable, S= swab detectable only, M= frank menstruation. [1]

	Days									
	0	1	2	3	5	7	6	7	8	9
ZK 137 316	**ZK Treatment ---------->**									
0.01 mg/kg (n=6)	U	U	U	U	U	U	U	U	U	U
0.03 mg/kg (n=6)	U	U	U	**S [1]**	**S [6]**	**S [6]**	U	U	U	U
0.05 mg/kg (n=4)	U	U	U	**S [4]**	**S [4]**	**S [3]** **M [1]**	**S [4]**	U	U	U
0.1 mg/kg (n=6)	U	U	U	**S [3]**	**S [6]**	**S [3]** **M [3]**	**S [5]** **M [1]**	U	U	U
0.15 mg/kg (n=3)	U	U	U	**S [1]**	**M [3]**	**M [3]**	**M [3]**	**M [2]**	**S [1]**	U
ZK 230 211										
0.005 mg/kg (n=2)	U	U	U	U	U	U	U	U	U	U
0.01 mg/kg (n=4)	U	U	U	U	**M [1]**	**S[1]**	U	U	U	U
0.03 mg/kg (n=4)	U	U	U	**S [4]**	**M [4]**	**M [4]**	**S [4]**	U	U	U
0.05 mg /kg (n=3)	U	U	**S [3]**	**M [3]**	**M [3]**	**M [3]**	**M [3]**	U	U	U
0.1 mg /kg (n=2)	U	U	**S [2]**	**S [2]**	**M [2]**	**M [2]**	**M [2]**	**M [2]**	**S [2]**	U

[1] Macaques were treated sequentially with implants of E_2 and then E_2 + P to create an artificial menstrual cycle. After 14 days of E+P priming, the animals were injected daily (im) with ZK 137 316 or ZK 230 211 for 7 days while the P implant remained in place. Numbers in brackets indicate the number of monkeys within each group that were swab positive or frankly menstruating.

Table 2. Menstrual blockade: bleeding patterns after P withdrawal in artificially cycled macaques treated continuously with ZK 316. U=undetectable, S= swab detectable only, M= frank menstruation. [1]

	Days after P withdrawal									
	0	1	2	3	4	5	6	7	8	9
Control (n=4)	U	U	M [2]	M [4]	M [4]	M [2]	U	U	U	U
0.01 mg/kg (n=3)	U	U	M [1]	M [3]	M [3]	M [1]	U	U	U	U
0.03 mg/kg (n=3)	U	U	S [1]	S[1]	S [2] M [1]	S [1]	U	U	U	U
0.05 mg/kg (n=3)	U	U	U	U	U	U	U	U	U	U
0.1 mg/kg (n=3)	U	U	U	U	U	U	U	U	U	U

[1]Macaques were injected daily (im) with during sequential treatment with E_2 and E_2 + P to create an artificial menstrual cycle. After 28 days the P implant was withdrawn and the ZK137 316 injections were continued through day 7 of the next cycle (shown in gray). Numbers in brackets indicate the number of monkeys within each group that were either swab positive or frankly menstruating.

Table 3. Reproductive tract morphometrics (Mean ± SE) in macaques treated with ZK 230 211. Means in each column with different superscripts are statistically different.

	E_2 alone (n=5)	E_2 + P (n=5)	E_2 + P + 0.005 mg ZK (n=3)	E_2 + P + 0.016 mg ZK (n=3)	E_2 + P + 0.032 mg ZK (n=3)
Endometrial Thickness mm)	3.4 ± 0.38^{a}	4.5 ± 0.55^{b}	3.01 ± 0.63^{ab}	2.2 ± 0.28^{c}	2.0 ± 0.22^{c}
Endometrial Mass (g) [1]	0.35 ± 0.03^{a}	0.41 ± 0.01^{b}	0.29 ± 0.08^{ab}	0.08 ± 0.03 ^{c}	0.07 ± 0.02 ^{c*}
Myometrial Mass (g) [1]	1.49 ± 0.20^{a}	1.15 ± 0.15^{a}	1.22 ± 0.28^{a}	1.51 ± 0.26^{a}	1.23 ± 0.35^{a}
Functionalis Ki-67 (%)	39.0 ± 4.0^{a}	1.0 ± 0.0^{b}	2.1 ± 1.1^{b}	28 ± 10.0 ^{ac}	26.0 ± 4.0^{c}
Functionalis Mitotic index [2]	18.5 ± 6.0^{a}	nd	nd	5.21 ± 0.7^{b}	3.23 ± 0.37 ^{b}
Basalis Ki-67 (%)	3.1 ± 2.0^{a}	39.0 ± 4.0^{b}	19.0 ± 8.0^{b}	3.1 ± 1.0^{a}	1.0 ± 1.0^{a}
Basalis Mitotic Index	0.88 ± 0.22^{a}	5.23 ± 1.12^{b}	3.16 ± 2.4^{b}	0.26 ± 0.07^{c}	0.15 ± 0.06^{c}
Functionalis Apoptotic cells (%)[3]	3.6 ± 1.03^{a}	0.17 ± 0.26^{b}	0.16 ± 0.37^{b}	3.32 ± 0.88^{a}	3.14 ± 1.24^{a}
Stromal Compaction [4]	69.3 ± 10.3^{a}	38.3 ± 9.4^{a}	95.6 ±8.7^{b}	167.4 ± 17.8^{c}	166.3 ± 8.7^{c}
Oviductal Weight (mg)	453 ± 97 ^{a}	400 ± 32^{a}	433 ± 29^{a}	443 ± 117 ^{a*}	327 ± 96^{a*}
Fimbrial Ciliation (%)	43.3 ± 8.8^{a}	4.7 ± 2.0^{b}	6.1 ± 2.5^{b}	39.0 ± 5.5^{a*}	42.3 ± 3.8^{a*}

[1] Endometrial and myometrial weights presented represent the weight from one-half of the uterus.

[2] Mitotic index represents the number of mitotic cells/ 1000 cells counted.

[3] Percent apoptotic cells was calculated from counts on 1200-5000 epithelial cells functionalis zone.

[4] Stromal compaction represents the number of stromal nuclei/10,000 um^2 at 400X magnification.

* n=2

Table 4. Effect of ZK 137 316 and ZK 230 211 on menstrual cycle lengths in rhesus monkeys[1].

	Pretreatment cycle lengths	Treatment-induced intermenstrual intervals	Recovery Period [3]	Post-treatment cycle lengths
Control [2] (n=8)	28.1 ± 1.0[a]	27.5 ± 0.85[a]	--	27.7 ± 1.1[a]
ZK 137 316 (40 day regimen)				
0.05 mg/kg (n=9)	31.2 ± 1.2[a]	55.0 ± 5.5[b]	15 ± 5.5	30.2 ± 5.2[a]
0.10 mg/kg (n=9)	30.1± 1.3[a]	61 ± 2.2[b]	21 ± 2.2	32.1 ± 2.3[a]
ZK 137 316 (100 day regimen)				
0.05 mg/kg (n=4)	31.6 ± 1.5[a]	131 ± 10.1[b]	31 ± 10.1	28.7 ± 0.8[a]
0.10 mg/kg (n=4)	29.6 ± 3.6[a]	134 ± 8.7[b]	34 ± 8.7	30.8 ± 1.5[a]
ZK 230 211 (60 day regimen)				
0.005 mg/kg (n=5)	27.4 ± 0.51[a]	27 ± 0.78[a]	0	29.2 ± 2.6[a]
0.016 mg/kg (n=5)	27.6 ± 1.0[a]	101 ± 7.4[b]	41 ± 7.4	55.2 ± 12.2[b]
0.050 mg/kg (n=5)	27.0 ± 2.0[a]	98 ± 5.9[b]	38 ± 5.9	38.2 ± 11.1[a]

[1]Values represent mean (± SE) intermenstrual interval in days. Means with different superscripts are statistically different (P<0.001).

[2]There was no effect on mean cycle length of injecting vehicle alone for 40 or 100 days. These data have therefore been combined as control.

[3]Recovery period was calculated from the end of treatment to the onset of the first post-treatment menses.

Table 5. Summary of the action of ZK 137 316 and ZK 230 211 on ovarian function and menstrual cycles in rhesus monkeys[1].

	E_2 surges	Luteal phases	Menstruation
Control [2] (n=8)	8	8	8 (Frank Menses)
ZK 137 316 (40 day regimen)			
0.05 mg/kg (n=9)	5	5	4 (minute bleeding)
0.10 mg/kg (n=9)	3	2	2 (minute bleeding)
ZK 137 316 (100 day regimen)			
0.05 mg/kg (n=4)	4	4	0
0.10 mg/kg (n=4)	0	0	0
ZK 230 211 (60 day regimen)			
0.005 mg/kg (n=5)	5	5	5 (Frank Menses)
0.016 mg/kg (n=5)	5	0	0
0.05 mg/kg (n=5)	0	0	0

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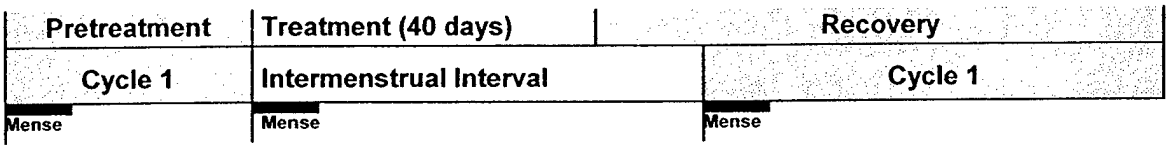
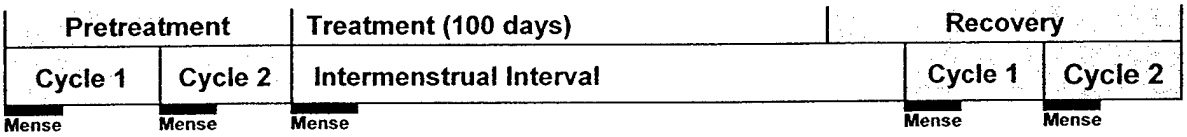
55 rhesus monkeys with low doses of the antiprogestin ZK 137 316: establishment of a
56 regimen that permits normal menstrual cyclicity. *Human Reproduction*, **13**, 259-267.

Figure 1 Top

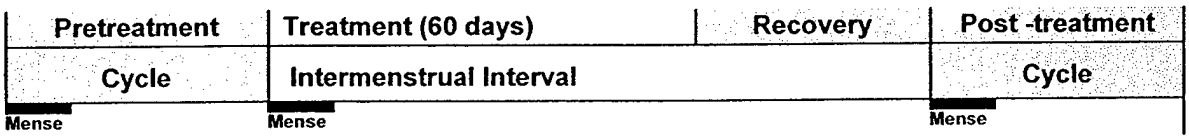
Experimental design 1: Short-term treatment with ZK 137 316

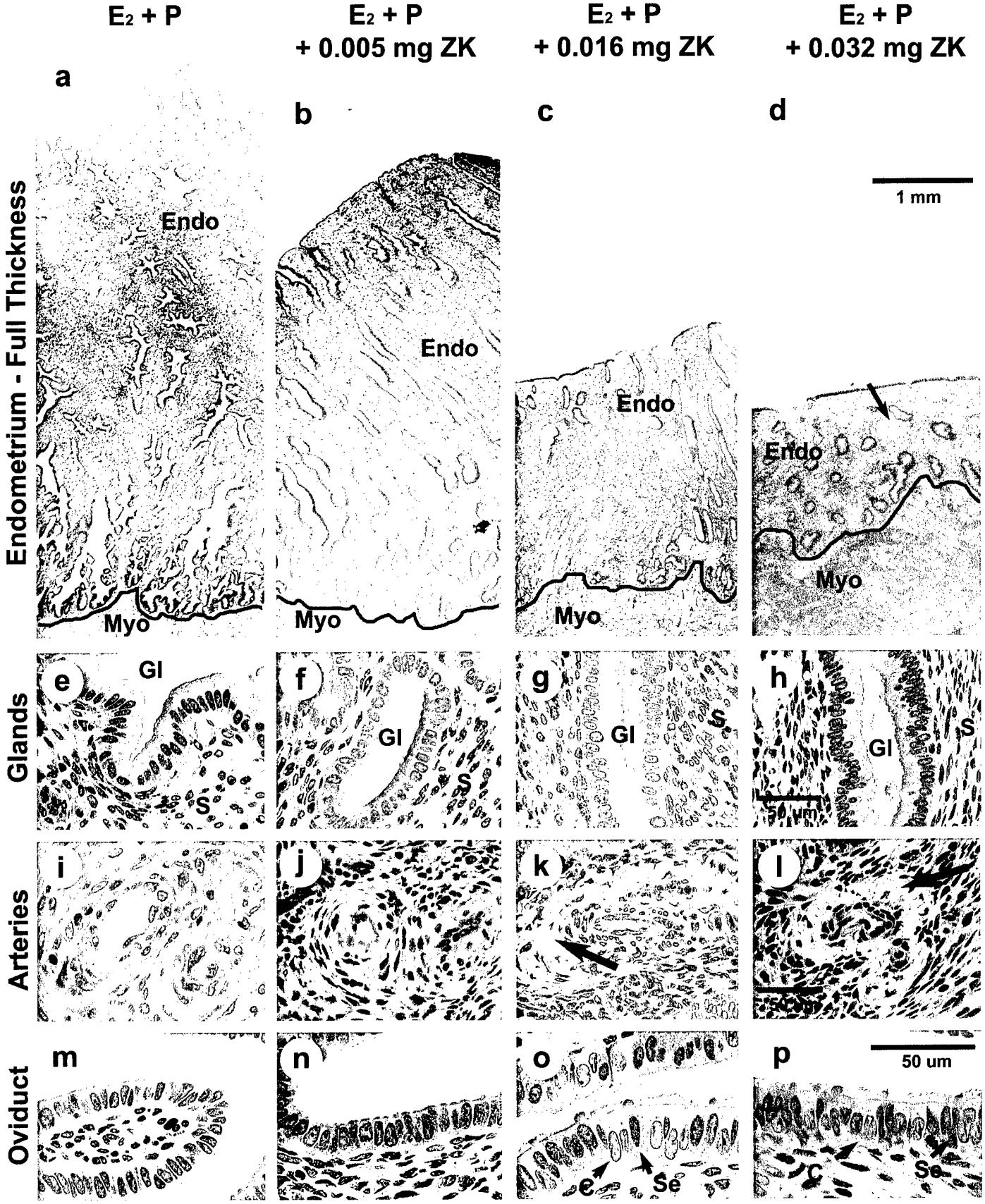


Experimental design 2: Long-term treatment with ZK 137 316



Experimental design 3: Intermediate treatment with ZK 230 211




E2 + P
E2 + P + 0.005 mg ZK
E2 + P + 0.016 mg ZK
E2 + P + 0.032 mg ZK
a
b
c
d
Endometrium - Full Thickness
Endo
Myo
1 mm
Glands
e
f
g
h
Gl
S
50 um
Arteries
i
j
k
l
Oviduct
m
n
o
p
e
Se
50 um

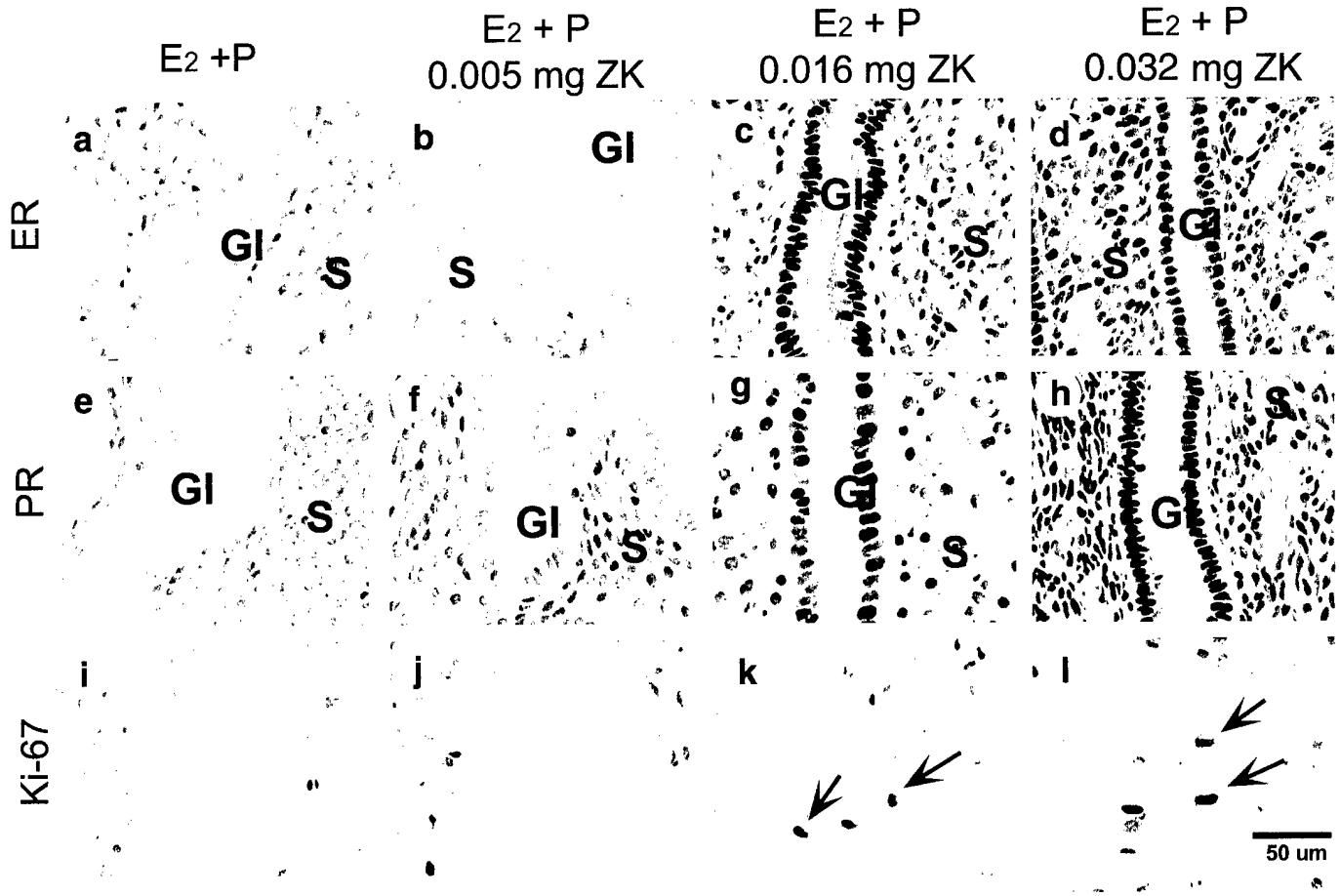

E2 +P
E2 + P
0.005 mg ZK
E2 + P
0.016 mg ZK
E2 + P
0.032 mg ZK
ER
PR
Ki-67
a
b
c
d
e
f
g
h
i
j
k
l
Gl
S
G
50 um

Figure 4 Top ↑

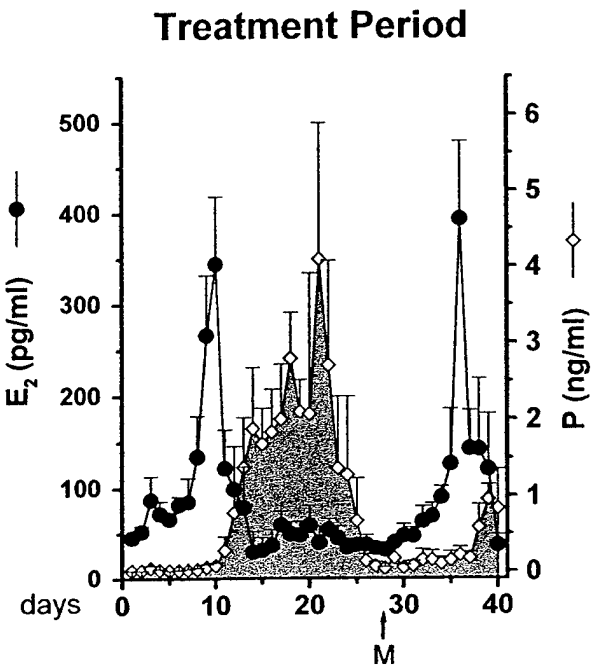

Treatment Period
E2 (pg/ml)
P (ng/ml)
500
400
300
200
100
0
6
5
4
3
2
1
0
days
0
10
20
30
40
M

Figure 5 Top

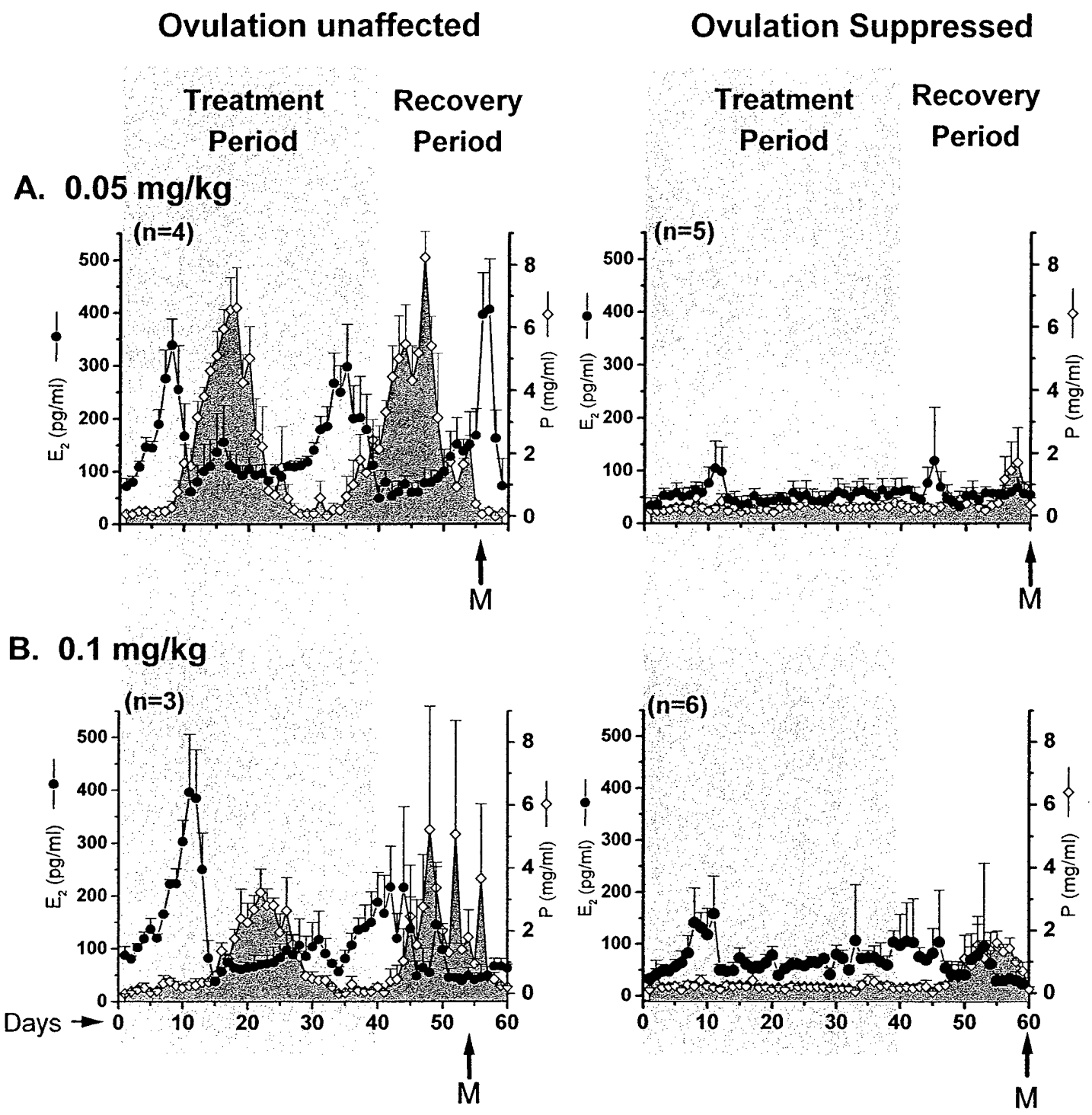

Ovulation unaffected
Ovulation Suppressed
Treatment Period
Recovery Period
Treatment Period
Recovery Period
A. 0.05 mg/kg
(n=4)
(n=5)
B. 0.1 mg/kg
(n=3)
(n=6)
E2 (pg/ml)
P (mg/ml)
Days
M

Figure 6 Top

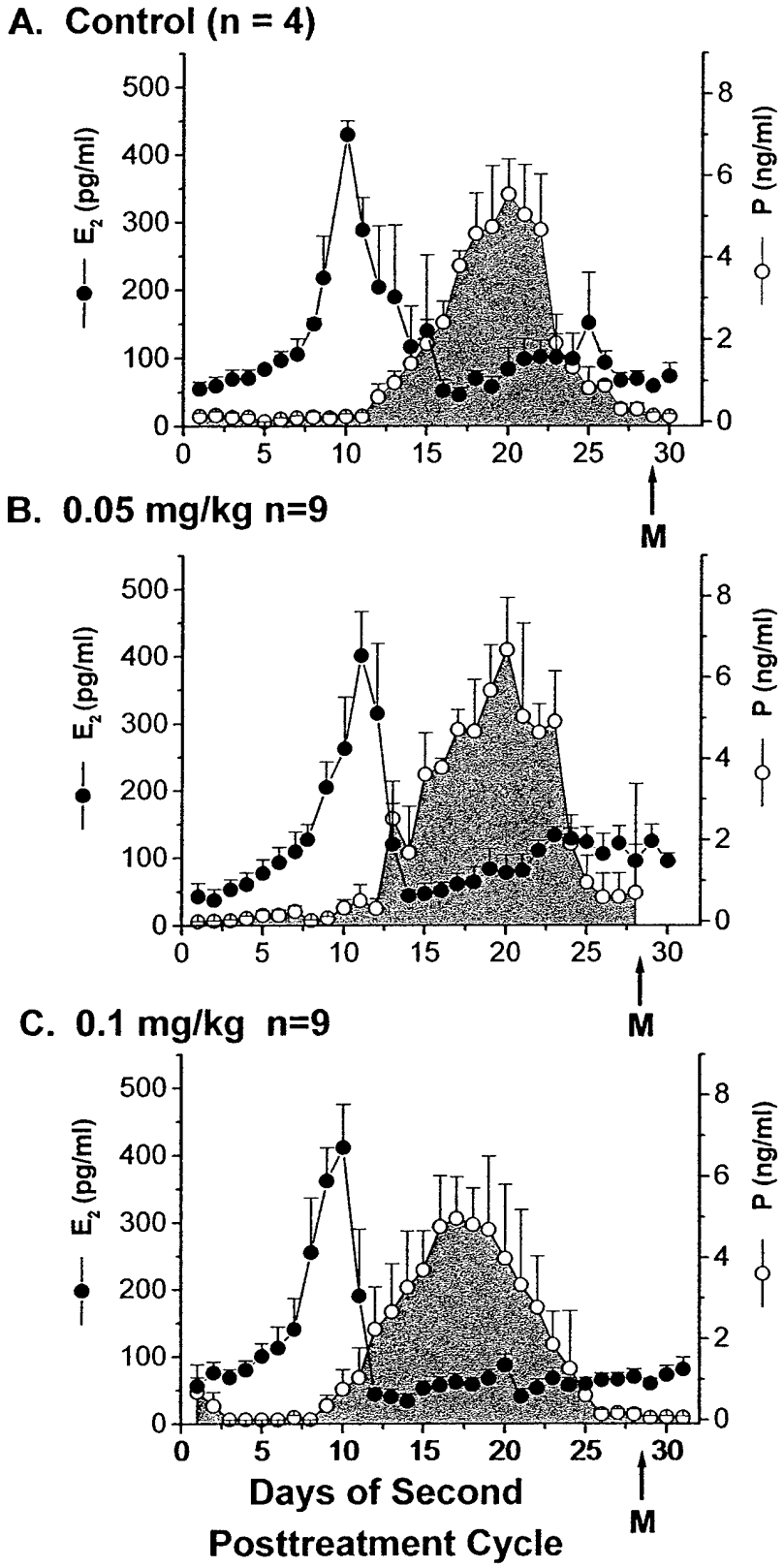
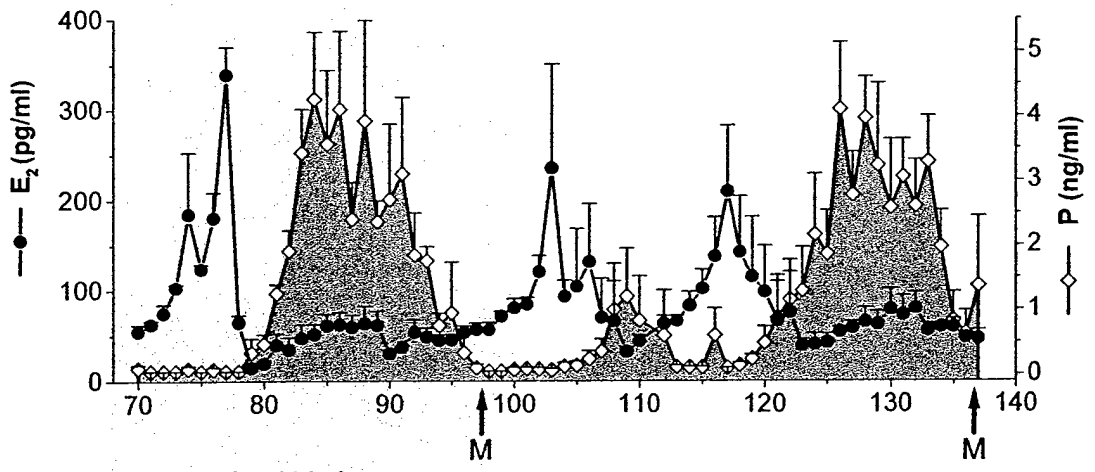

A. Control (n = 4)
B. 0.05 mg/kg n=9
C. 0.1 mg/kg n=9
E2 (pg/ml)
P (ng/ml)
M
Days of Second Posttreatment Cycle

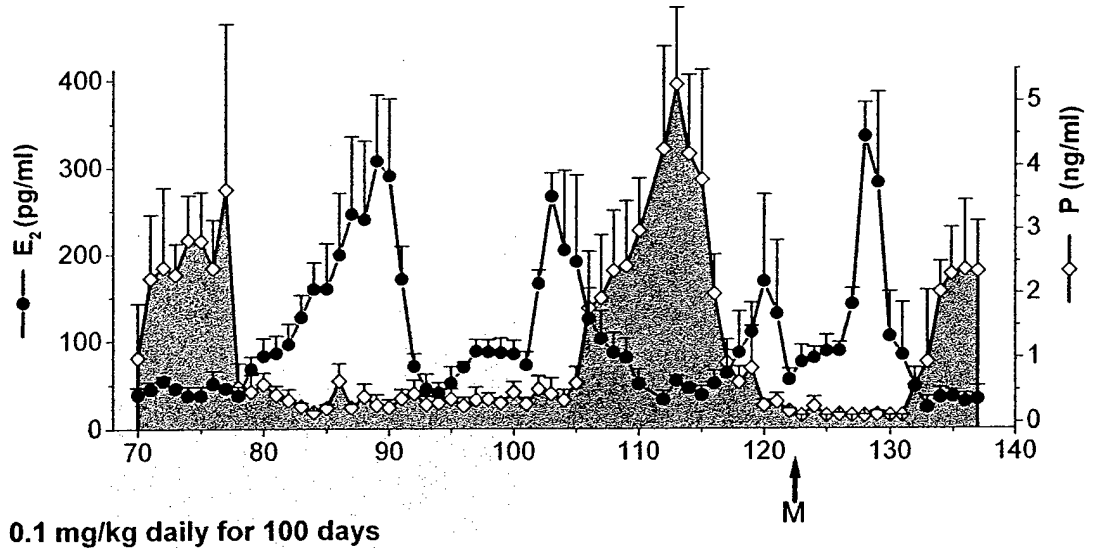
Figure 7 Top

Last day of treatment

A. Vehicle Control



B. 0.05 mg/kg daily for 100 days



C. 0.1 mg/kg daily for 100 days

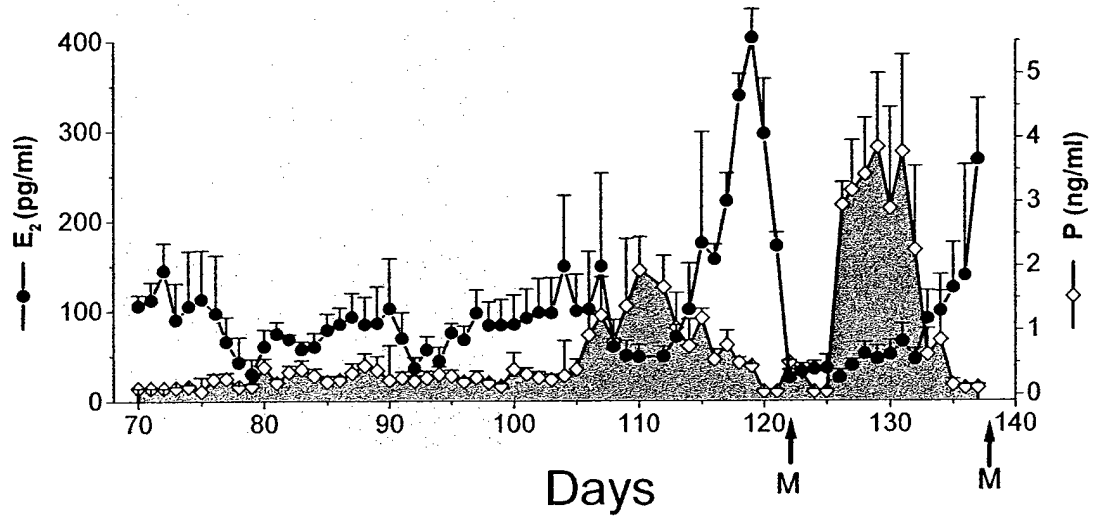


Figure 8 Top

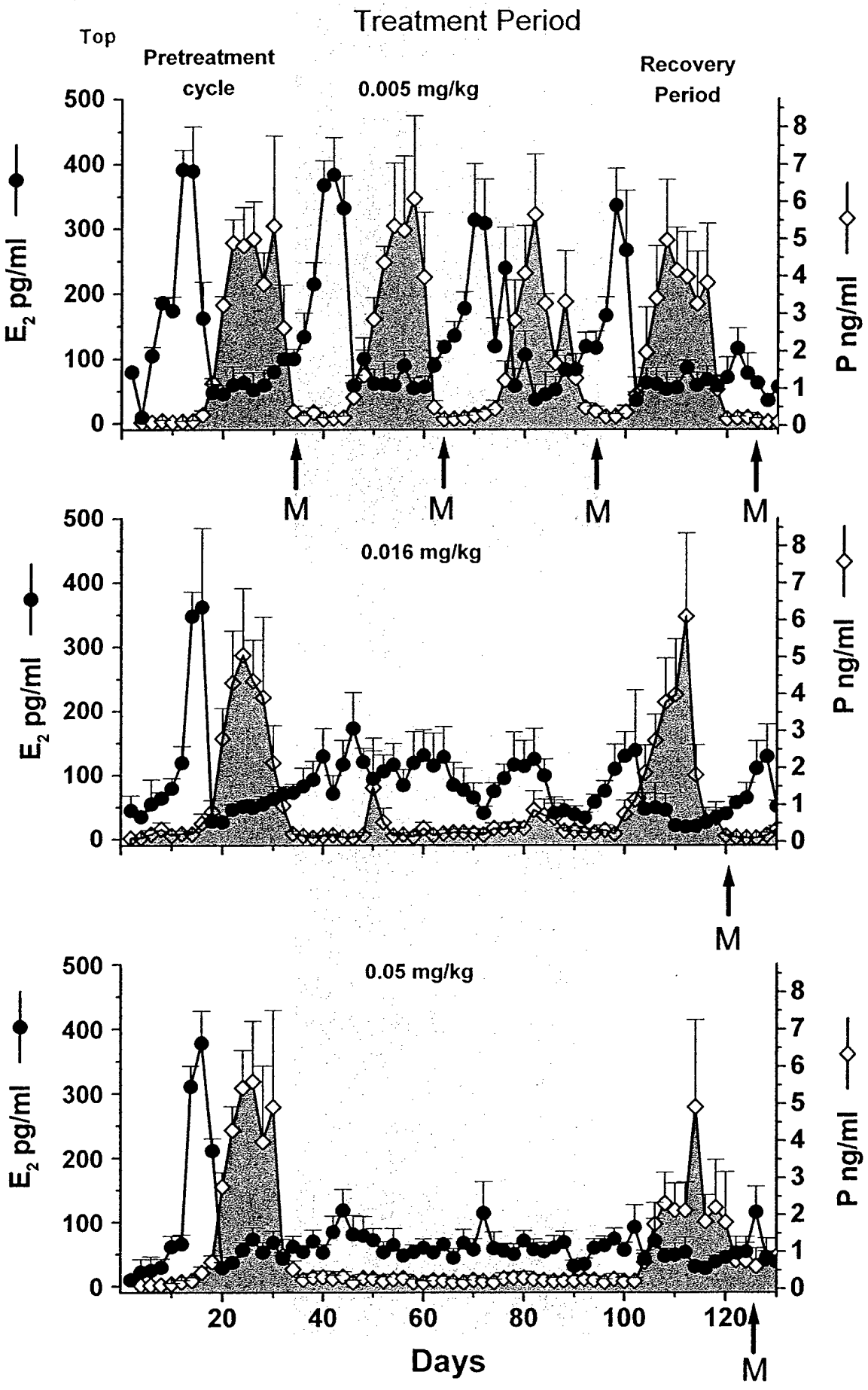
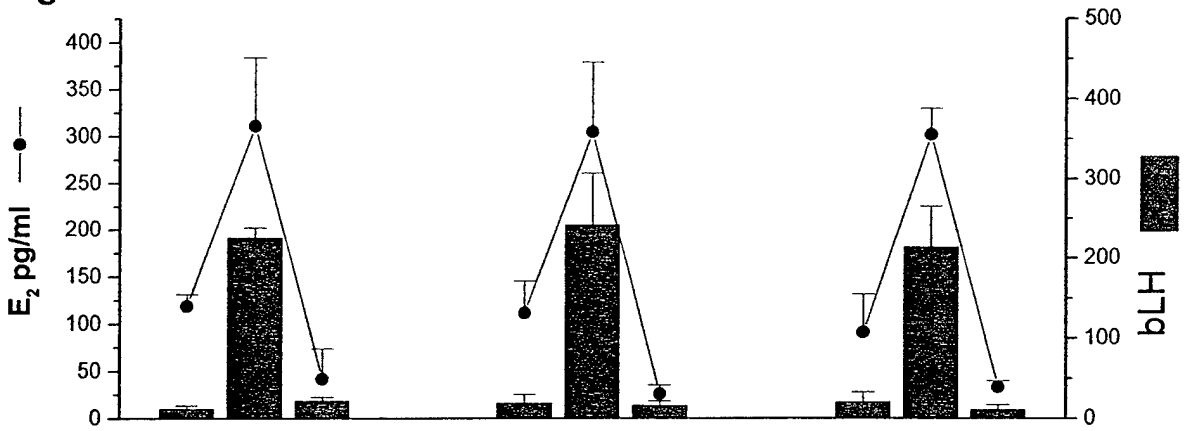
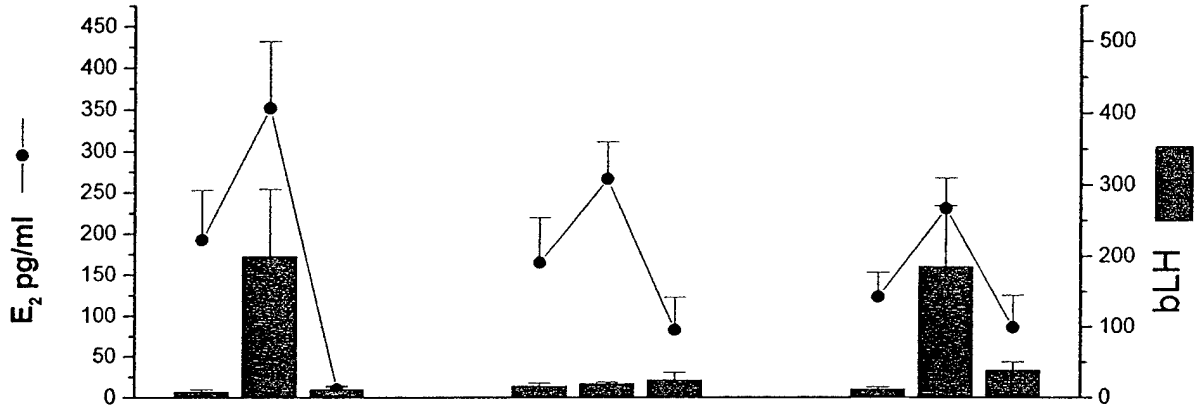

Treatment Period
Pretreatment cycle
0.005 mg/kg
Recovery Period
0.016 mg/kg
0.05 mg/kg
E2 pg/ml
P ng/ml
M
Days
0
100
200
300
400
500
1
2
3
4
5
6
7
8
20
40
60
80
100
120

Figure 9 Top

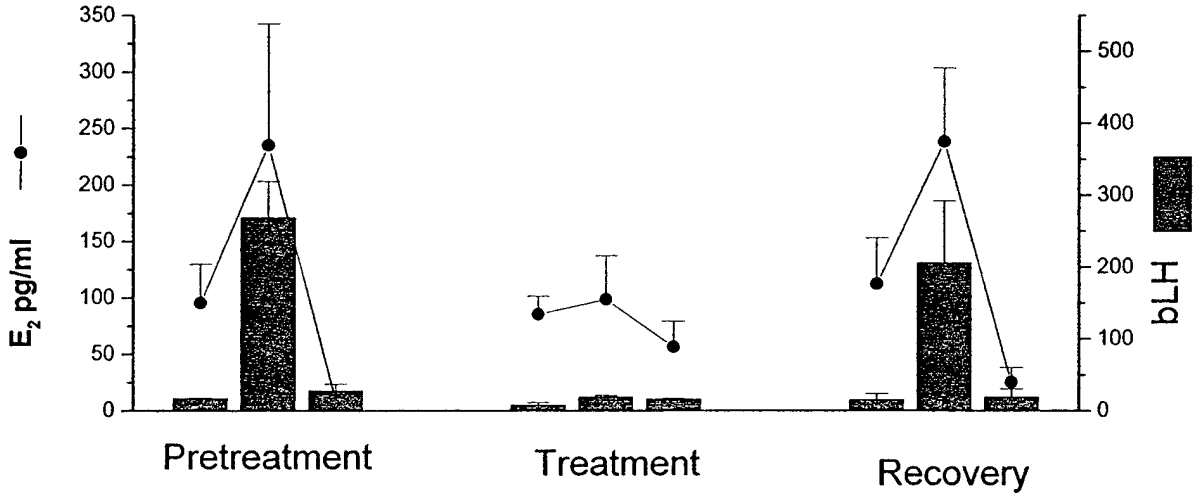
A. 0.005 mg ZK 230 211



B. 0.016 mg ZK 230 211



C. 0.05 mg ZK 230 211



Human Reproduction vol.13 no.2 pp.259–267, 1998

OUTSTANDING CONTRIBUTION

Chronic treatment of female rhesus monkeys with low doses of the antiprogestin ZK 137 316: establishment of a regimen that permits normal menstrual cyclicity

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Large doses of antiprogestin typically disrupt menstrual cyclicity. A chronic low-dose regimen of the potent new antiprogestin ZK 137 316, which permits continued menstrual cyclicity but alters gonadal–reproductive tract activity, was established. Rhesus monkeys received vehicle (n = 6) or 0.01 (n = 8), 0.03 (n = 8) or 0.1 (n = 5) mg ZK 137 316/kg body weight daily for five menstrual cycles (C-1 to C-5). Oestradiol, progesterone and gonadotrophin profiles were normal during cycles involving vehicle and 0.01 and 0.03 mg ZK 137 316/kg body weight. In the 0.1 mg/kg group, mid-cycle oestradiol and gonadotrophin surges, and subsequent progesterone production, were absent in C-3 and C-5. Ovarian cyclicity was accompanied by timely menstruation in the vehicle and 0.01 mg/kg groups. By C-3, half the animals in the 0.03 mg/kg group and all animals in the 0.1 mg/kg group were amenorrhoeic. A corpus luteum was noted during the mid-luteal phase of C-5 in the vehicle, 0.01 mg/kg and 0.03 mg/kg groups. Large antral and cystic follicles were evident in the 0.1 mg/kg group. Thus, a daily treatment with 0.01 mg/kg ZK 136 317 permitted normal menstrual cyclicity in macaques. While the daily administration of 0.03 mg/kg ZK 136 317 allowed ovarian cyclicity, menstruation was disrupted in some animals. Increasing the dose to 0.1 mg/kg antagonized pituitary function and resulted in anovulation and amenorrhoea. A chronic low-dose regimen of the antiprogestin ZK 137 316, which permits normal ovarian/menstrual cyclicity, has potential as a contraceptive in women.

Key words: antiprogestin/menstrual cycle/ovary/steroids/ZK 137 316

Introduction

Recent methods of fertility regulation using antiprogestins in women and monkeys have focused on the acute administration of large doses of RU 486 (mifepristone; Roussel Uclaf, Romainville, France; Lebeau and Baulieu, 1994) or ZK 98 299 (onapristone; Schering AG, Berlin, Germany; Puri and Van Look, 1991) at specific stages of the menstrual cycle. Large doses of antiprogestin (i) disrupt development of the dominant follicle or prevent the luteinizing hormone (LH) surge during the mid- or late follicular phase; (ii) induce premature menstruation in the mid-luteal phase; or (iii) prevent pregnancy with late luteal phase administration (reviewed by Van Look and von Hertzen, 1995). While these regimens would impair fertility in the treatment interval (Ghosh *et al.*, 1997), the feasibility of once-a-month administration of antiprogestin as a general contraceptive remains to be substantiated.

Chronic treatment with low doses of antiprogestin may represent a new mode of regulating fertility in women. This is supported by observations that very low doses (e.g. 0.1–1.0 mg/kg) of antiprogestins can retard maturation of the human (Batista *et al.*, 1992; Kettel *et al.*, 1992; Croxatto *et al.*, 1993; Cameron *et al.*, 1995, 1996; Gemzell-Danielsson *et al.*, 1996) or non-human primate (Ishwad *et al.*, 1993; Katkam *et al.*, 1995) endometrium without causing premature menstruation or greatly disturbing menstrual cyclicity. Although blockade of pregnancy was not an end-point in clinical studies, low doses of antiprogestins given to laboratory animals throughout the ovarian cycle inhibit ovulation and/or implantation in a dose-dependent manner (Vinijsanun and Martin, 1990; Batista *et al.*, 1991; Roblero and Croxatto, 1991).

The aim of this study was to establish a daily chronic treatment regimen with low doses of a new potent antiprogestin, ZK 137 316 (Schering AG), in rhesus monkeys that will permit continued ovarian/menstrual cyclicity but alter gonadal and/or reproductive tract function to inhibit fertility. This study will provide the basis for evaluating the usefulness of chronic low-dose ZK 137 316 as a potential contraceptive.

Materials and methods

Animals and treatments

Adult female rhesus monkeys were caged individually under controlled conditions of temperature (22°C) and a standard daily light–dark cycle (12L:12D). Acute dose-ranging studies were performed on three ovariectomized rhesus monkeys during artificial menstrual cycles (Slayden *et al.*, 1993) established with sequential implants of oestradiol (2–3 weeks) followed by oestradiol plus progesterone (2 weeks). After 1 week of oestradiol + progesterone exposure, animals received the antiprogestin ZK 137 316 (Schering AG), a new antiprogestin with an increased antiprogestagenic potency and

reduced antiglucocorticoid activity (unpublished data; Schering AG) at 0.03 and 0.1 mg/kg body weight (i.m., once daily for 4 days) to determine the highest dose that would permit cyclicity. Treatments with these doses of ZK 137 316 were repeated in two successive artificial cycles in the same animals. Menstruation was consistently induced with 0.1 mg/kg within 24 h of the last injection, but not with 0.03 mg/kg. To confirm these results during the normal menstrual cycle, 0.03 and 0.1 mg/kg ZK 137 316 (n = 3 in each group) were administered (i.m., once a day) for 3 days during the mid-luteal phase (7–8 days after the mid-cycle oestradiol surge) to monkeys during natural cycles. Premature menstruation was observed in two out of three animals within 48 h of the last 0.1 mg/kg injection. However, all of the animals treated with 0.03 mg/kg exhibited timely, but not premature, menstruation.

Based on these results, adult female rhesus monkeys (n = 27) exhibiting regular menstrual cycles were assigned randomly to receive a single daily i.m. injection of vehicle (controls; n = 6; 25% ethanol/37.5% propylene glycol/37.5% saline, v/v/v; 0.5 ml) or 0.01 (n = 8), 0.03 (n = 8) or 0.1 (n = 5) mg ZK 137 316/kg body weight in vehicle. Animals were monitored for seven consecutive cycles between October 1995 and June 1996. The first cycle of the series served as a pretreatment cycle. During the second cycle, animals did not undergo any experimental procedures. Beginning with the third cycle, daily injections of vehicle or ZK 137 316 were initiated and continued for five consecutive cycles.

Monkeys in each group were checked daily for menses during all cycles, and the duration of menstruation was recorded. A cycle was defined as the interval from menstruation to menstruation. If menstruation was absent or not detected, but functional follicular and luteal phases based on steroid hormone concentrations were observed, the duration of the luteal phase was recorded as the number of days that serum progesterone concentrations were ⩾1.0 ng/ml. If both menstruation and ovarian activity were absent, 'cycles' were designated as ~30 day intervals.

Hormone determinations

Saphenous venous blood samples were collected daily during the pretreatment cycle and treatment cycles 1, 3 and 5. Serum was analysed for oestradiol and progesterone by the Endocrine Services Laboratory at Oregon Regional Primate Research Center (Beaverton, OR, USA) using radioimmunoassays established for macaque serum (Resko *et al.*, 1974, 1975). Bioactive LH was measured in serum samples from treatment cycles 1 and 5 using a mouse Leydig cell bioassay, validated previously for macaque serum (Ellinwood *et al.*, 1984), with cynomolgus LH as the standard. Concentrations of circulating follicle stimulating hormone were also determined during treatment cycles 1 and 5 by a homologous radioimmunoassay validated for macaque serum using reagents and procedures provided by the Center for Population Research (NICHD, Bethesda, MD, USA) and A.F.Parlow (Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center, Torrance, CA, USA). Recombinant cynomolgus monkey (rcm) FSH (AFP 6940A) was used as the standard and iodinated antigen, and the antibody was rabbit anti-rcmFSH (AFP782594). Assay of multiple dilutions of a rcmFSH solution (0.1–10 ng), as well as serum from ovariectomized or intact amenorrhoeic macaques and from monkeys during the follicular phase of normal menstrual cycles (20–400 µl), resulted in displacement curves parallel to the standard curve. The sensitivity of the assay was 0.38 ng/ml using 100 µl of serum. FSH was undetectable in serum from hypophysectomized female macaques. The intra- and interassay coefficients of variation for the FSH radioimmunoassay, as determined in a serum pool from ovariectomized macaques, were 9.5 (n = 9 separate assays) and 22% (n = 7 separate assays) respectively.

Ovarian characteristics

At the mid-luteal phase of treatment cycle 5, an ovariohysterosalpingectomy was performed (n = 3 per group). Dominant ovarian structures were documented visually. Ovaries containing dominant structures were subsequently fixed in formalin, sectioned and stained with haematoxylin for histological evaluation. The reproductive tract (i.e. uterus and oviducts) was also prepared for histological and immunocytochemical analyses; the histological changes in the tract are presented in a separate manuscript (Slayden *et al.*, 1998).

Statistical analyses

When heterogeneity of variance was observed, data were transformed (logarithm + 10) prior to analyses. Steroid and gonadotrophin hormone concentrations obtained during treatment cycles 1, 3 and 5 were analysed by two-way analyses of variance (ANOVA) with one repeated measure, with comparisons among treatment groups made using Newman–Kuehl's test. One-way ANOVA with one repeated measure was used to analyse hormone concentrations over time within a treatment group, followed by Newman–Kuehl's test for comparisons among days. Data were considered to be statistically significant at $P < 0.05$.

Results

There was no difference in the total number of days (mean ± SEM) constituting the treatment interval among the groups (control, 150 ± 6; 0.01 mg/kg group, 143 ± 4; 0.03 mg/kg group, 145 ± 4; 0.1 mg/kg group, 143 ± 4).

Circulating concentrations of oestradiol and progesterone in vehicle-treated animals during treatment cycles 1, 3 (data not shown) and 5 (Figure 1) were within the 95% confidence interval of values obtained from all 27 animals during the pretreatment cycle of the study (Figure 1). Likewise, similar profiles of serum oestradiol and progesterone concentration were observed during treatment cycles 1, 3 (data not shown) and 5 (Figure 1) in the 0.01 and 0.03 mg/kg groups relative to the pretreatment cycle. All five animals in the 0.1 mg/kg group exhibited normal oestradiol profiles during the follicular phase of treatment cycle 1, but only two out of five animals displayed normal concentrations of serum progesterone indicative of a functional corpus luteum (Figure 2). In the 0.1 mg/kg group, menstruation was observed in one out of two and in one out of three animals with normal and deficient luteal phases respectively during treatment cycle 1. By cycle 3 in the 0.1 mg/kg treatment, oestradiol concentrations remained typical of the early and mid-follicular phases in untreated animals, but peak concentrations were suppressed. Serum progesterone concentrations were <1.0 ng/ml following the peak oestradiol concentration, and menstruation was absent in all animals. Similar patterns of oestradiol and progesterone concentration were observed during treatment cycles 3 and 5 with 0.1 mg/kg ZK 137 316 (Figure 2).

Timely LH and FSH surges of a magnitude similar to that of control animals were observed during treatment cycles 1 (data not shown) and 5 (Figure 3) in the 0.01 and 0.03 mg/kg groups. Likewise, LH and FSH concentrations during the follicular and luteal phases were similar among the control,

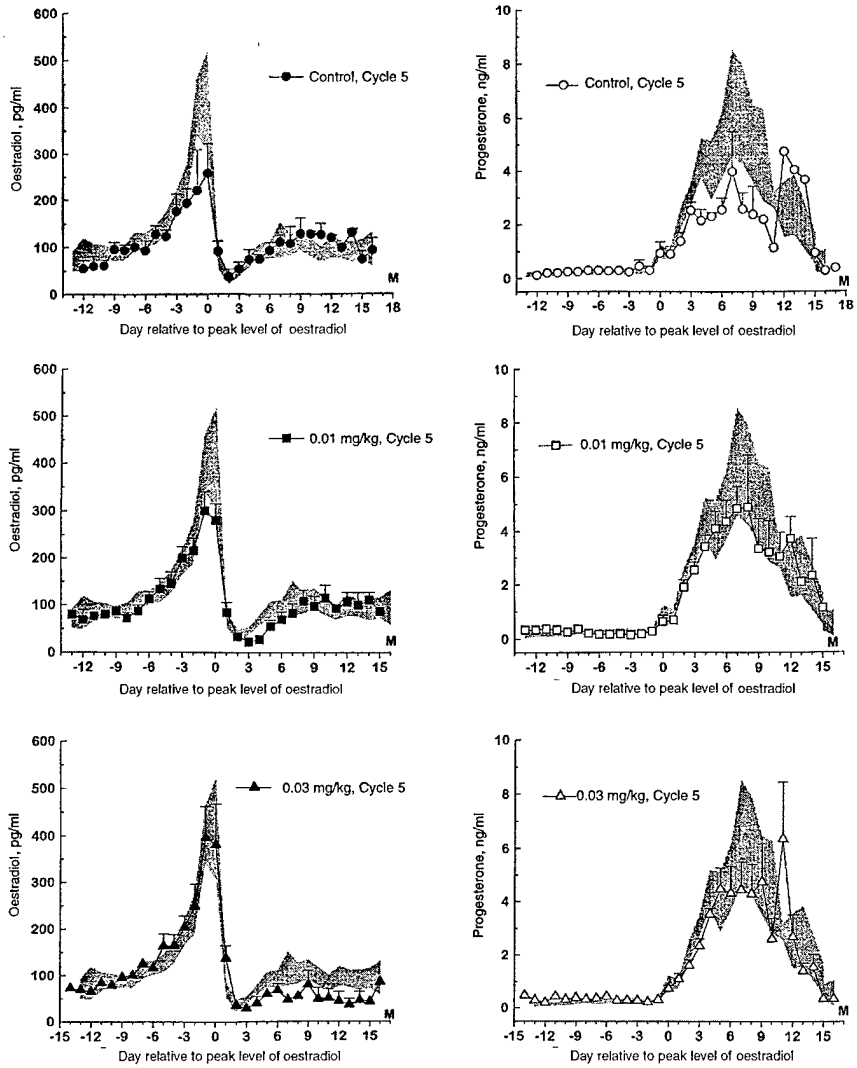

Figure 1. Serum oestradiol (left panel) and progesterone (right panel) concentrations in animals receiving vehicle (controls, $n = 5$) or 0.01 ($n = 7$) and 0.03 ($n = 6$) mg ZK 137 316/kg body weight during cycle 5 of treatment. Each point represents the mean ± SEM value of animals displaying ovarian activity (see Table I). The shaded area represents the 95% confidence interval for oestradiol or progesterone concentrations observed in all animals during the pretreatment cycle. The data are plotted relative to the day the peak concentration of oestradiol was observed, designated day 0. The mean day of menstruation is indicated by 'M'.

0.01 and 0.03 mg/kg groups. By contrast, both LH and FSH surges were absent in animals not exhibiting luteal phases in the first (Figure 4) and fifth (Figure 3) cycle of 0.1 mg/kg ZK 137 316 treatment. In the 0.1 mg/kg group, LH and FSH concentrations during the follicular phase of treatment and LH concentrations during the luteal phase of treatment in cycles 1 (Figure 4) and 5 (Figure 3) were similar to those of the controls. In addition, FSH concentrations during the luteal phase of treatment cycle 1 in animals that received 0.1 mg/kg and exhibited a luteal phase were typical of controls (Figure 4). However, concentrations of FSH were higher ($P < 0.05$) than in controls on days 7–13 of cycle 1 (Figure 4) in animals treated with 0.1 mg/kg that did not display a luteal phase. Furthermore, elevated ($P < 0.05$) FSH concentrations were observed in all animals on days 4–11 after the peak oestradiol concentration was observed during cycle 5 in the 0.1 mg/kg treatment group compared with controls (Figure 3).

The majority of animals in the control (5/6), 0.01 mg/kg (7/8) and 0.03 mg/kg (6/8) groups continued to display ovarian cycles, based on serum hormone patterns, at the end of treatment cycle 5 (Table I). However, a smaller proportion of monkeys were cycling (2/5) in the 0.1 mg/kg group after cycle 1, and none of the animals treated with this dose exhibited normal ovarian activity by the end of treatment cycle 3. Continued suppression of ovarian cyclicity was noted in the 0.1 mg/kg group during treatment cycle 5. In the 0.1 mg/kg group, non-cycling animals typically did not menstruate at the end of any of the treatment cycles (Table I). While all control animals menstruated at the end of cycle 1, two out of seven, three out of seven and three out of five in the 0.01, 0.03 and 0.1 mg/kg groups respectively, did not menstruate at the end of cycle 1 despite displaying ovarian cycles. At the end of cycles 3 and 5 a greater proportion of animals (4/8) in the 0.03 mg/kg group did not menstruate relative to the control (1/6) and 0.01 mg/kg (2/8) groups. All animals in the 0.1 mg/kg

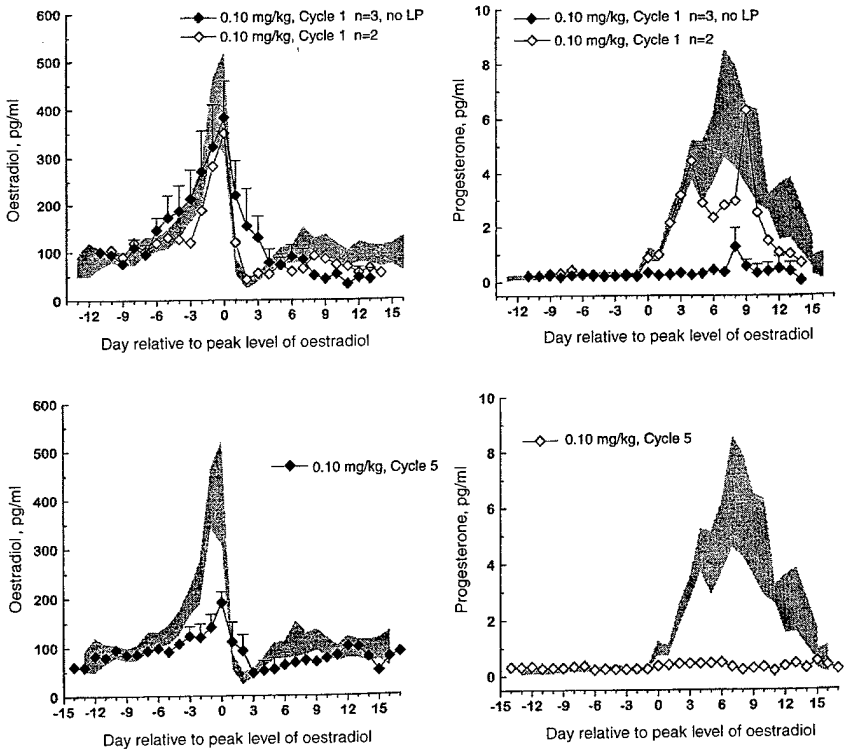

Figure 2. The pattern of oestradiol (left panel) and progesterone (right panel) concentrations during treatment cycles 1 (top) and 5 (bottom) in animals (n = 5/cycle) receiving daily injections of 0.1 mg/kg ZK 137 316. See the legend to Figure 1 for details.

group were amenorrhoeic by the start of treatment cycle 3. No evidence of 'breakthrough bleeding', i.e. overt menstruation at intervals <28–30 days typical of a normal menstrual cycle, was observed in any treatment group throughout antiprogestin treatment.

Within a treatment group there were no differences in the duration of the follicular and luteal phases and of the menstrual cycle over all treatment cycles (Table II). Likewise the lengths of the follicular phase, luteal phase and menstrual cycle were similar among animals displaying ovarian cycles in the control, 0.01 mg/kg and 0.03 mg/kg groups in treatment cycles 1, 3 and 5. Only one animal receiving 0.03 mg/kg displayed an abnormally long follicular phase (~24 days) during treatment cycle 5. In contrast, three out of five animals in the 0.1 mg/kg group did not exhibit luteal phases and hence ovarian cycles in cycle 1, and by treatment cycle 3 all animals in this group had ceased normal menstrual cyclicity with a lack of distinct follicular and luteal phases.

At the mid-luteal phase of treatment cycle 5, control animals had a corpus luteum on one ovary (Figure 5), with the contralateral ovary typically containing numerous small (~0.5 mm in diameter) follicles. Likewise, a corpus luteum was noted in each animal from the 0.01 mg/kg group and in two out of three animals in the 0.03 mg/kg group (Figure 5). Although a corpus luteum was present after 0.01 and 0.03 mg/kg ZK 137 316, some of them contained large central cavities. Histological examination of serial sections through these corpora lutea is underway to examine the potential for 'trapped', i.e. unovulated, oocytes as well as the occurrence of subnormal luteinization. The remaining animal in the 0.03 mg/kg group did not display menstruation at the start of treatment cycle 5. The ovaries were removed at what was estimated to be the mid-luteal phase, but subsequent steroid assays indicated that oestradiol concentrations continued to rise during what appeared to be an ~24 day follicular phase. Consistent with the oestradiol pattern, one ovary contained a 2 mm follicle and the other ovary had three 1 mm follicles.

None of the ovaries examined from animals in the 0.1 mg/kg group contained a corpus luteum. Ovaries from two animals in this group had many large antral follicles, ranging in diameter from 1 to 6 mm (Figure 5); the remaining animal displayed an extremely large (2.5×2.3 cm) ovarian cyst. While regressed corpora lutea, presumably from previous cycles, were typically evident in ovaries from the control, 0.01 mg/kg and 0.03 mg/kg groups, none were observed in the 0.1 mg/kg group.

Slayden *et al.* (1998) described the effects of chronic low doses of ZK 137 316 on endometrial morphology. Following vehicle treatment, the endometrium was in a hypertrophied, progestational (secretory) state consistent with the effects of progesterone during the mid-luteal phase. Daily treatment for 5 consecutive months with ZK 137 316 at all doses inhibited progestational differentiation of the endometrium relative to vehicle-injected controls (Slayden *et al.*, 1998). This effect was associated with increased stromal compaction, decreased number and sacculation of endometrial glands and an overall thinning of the endometrium. At the lowest dose of ZK 137 316 tested (0.01 mg/kg), the glandular epithelium of the functionalis zone appeared proliferative, but was reduced in height from columnar to low cuboidal. Treatment with 0.03 and 0.1 mg/kg ZK 137 316 resulted in further atrophy of

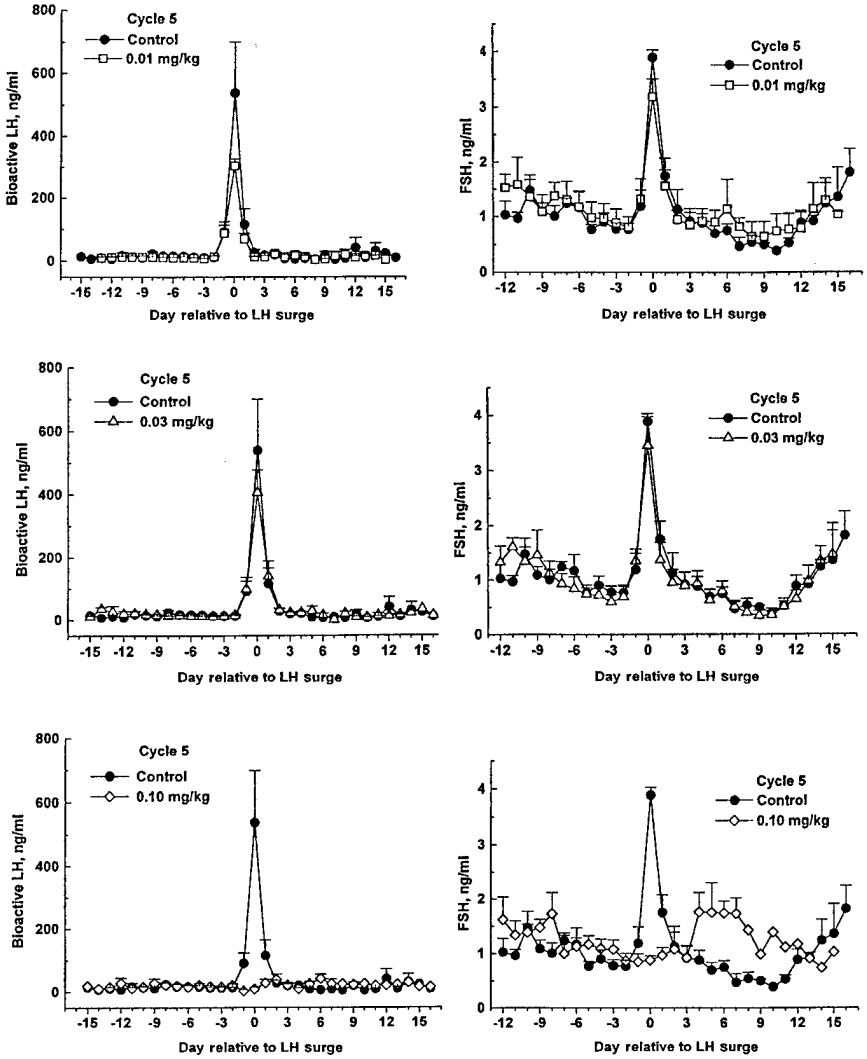

Figure 3. Concentrations of bioactive luteinizing hormone (LH; left panel) and immunoactive follicle stimulating hormone (FSH; right panel) during treatment cycle 5 in control animals (n = 5) and those receiving 0.01 (n = 7), 0.03 (n = 6) or 0.1 (n = 5) mg/kg ZK 137 316. Each point represents the mean ± SEM value of animals displaying ovarian cyclicity in the control, 0.01 and 0.03 mg/kg groups (see Table I), and for all animals in the 0.1 mg/kg group. Data are plotted relative to the day of the peak LH/FSH concentration (designated day 0) for the control, 0.01 and 0.03 mg/kg groups, and to the day of peak oestradiol concentration in the 0.1 mg/kg group. The acyclic animal in the 0.01 mg/kg group had a peak LH concentration of 70.98 ng/ml. A mid-cycle gonadotrophin surge was absent in two animals that received 0.03 mg/kg ZK 137 316 (see Table I).

the glandular epithelial cells in both the functionalis and basalis zones. Slayden *et al.* (1997) also observed degenerative hyalinization of the spiral arteries and dilation of the endometrial veins at all doses of ZK 137 316 relative to vehicle-treated controls.

Discussion

This study identifies, for the first time, low doses of the potent new antiprogestin ZK 137 316 that allow ovarian cyclicity without inducing amenorrhoea when administered daily to rhesus monkeys. Our study supports the concept of a daily low-dose antiprogestin regimen as a potential contraceptive strategy, as proposed initially by Batista *et al.* (1992) and confirmed recently by others (Kettel *et al.*, 1992; Croxatto *et al.*, 1993; Cameron *et al.*, 1995, 1996; Gemzell-Danielsson *et al.*, 1996). Low-dose daily treatment with antiprogestin offers many advantages relative to the acute administration of a large dose of antiprogestin at a specific stage of the menstrual cycle. Acute treatment during the follicular phase is effective in the treatment interval (Spitz *et al.*, 1996), but variability in the duration of the follicular phase would preclude critical timing of antiprogestin treatment in the subsequent cycle. Large doses of antiprogestin induce chronic anovulation, but accompanying unopposed oestradiol action raises concerns regarding endometrial, cervical or mammary gland hyperplasia (Katkam *et al.*, 1995; Cameron *et al.*, 1996). Once-a-month administration of antiprogestin during the early luteal phase relies on the accurate determination of the LH surge. If given too early, ovulation is suppressed with delayed onset of the next menstruation; if administered too late, menstruation may occur but an implanted embryo may fail to dislodge (Van Look and von Hertzen, 1995). Although a single dose (2 mg/kg) of mifepristone given 2 days post-ovulation

prevented pregnancy in rhesus monkeys (Ghosh *et al.*, 1997), late luteal phase administration was not an effective contraceptive in women (Couzinet *et al.*, 1990; Van Look and von Hertzen, 1994).

While the effectiveness of daily low-dose antiprogestin treatment (either mifepristone or onapristone) was assessed during a single menstrual cycle in the majority of clinical studies (Batista *et al.*, 1992; Kettel *et al.*, 1992; Croxatto *et al.*, 1993; Cameron *et al.*, 1995, 1996; Gemzell-Danielsson *et al.*, 1996), our data in the rhesus monkey indicate that ovarian/menstrual cyclicity was maintained throughout the chronic daily administration of 0.01 or 0.03 mg/kg ZK 137 316

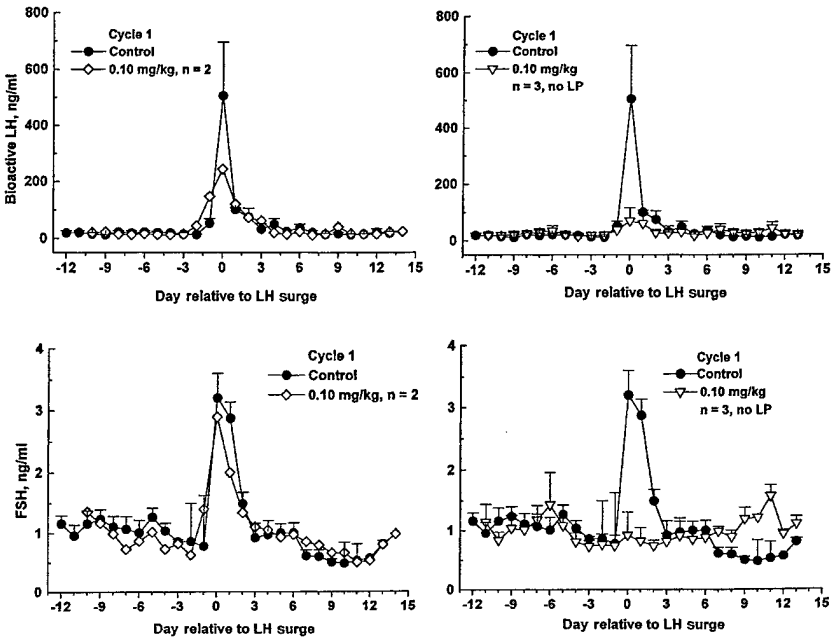

Figure 4. Concentrations of bioactive luteinizing hormone (LH; upper panel) and immunoreactive follicle stimulating hormone (FSH; lower panel) during treatment cycle 1 in animals receiving 0.1 mg/kg ZK 137 316. For the two animals exhibiting ovarian activity (left panel), each point represents the mean. For the remaining three animals that did not display a luteal phase (right panel), each point represents the mean ± SEM. Data are plotted relative to the day of the peak LH/FSH concentration (designated day 0) in animals displaying ovarian cyclicity and to the peak oestradiol concentration in the remaining animals that did not exhibit a luteal phase.

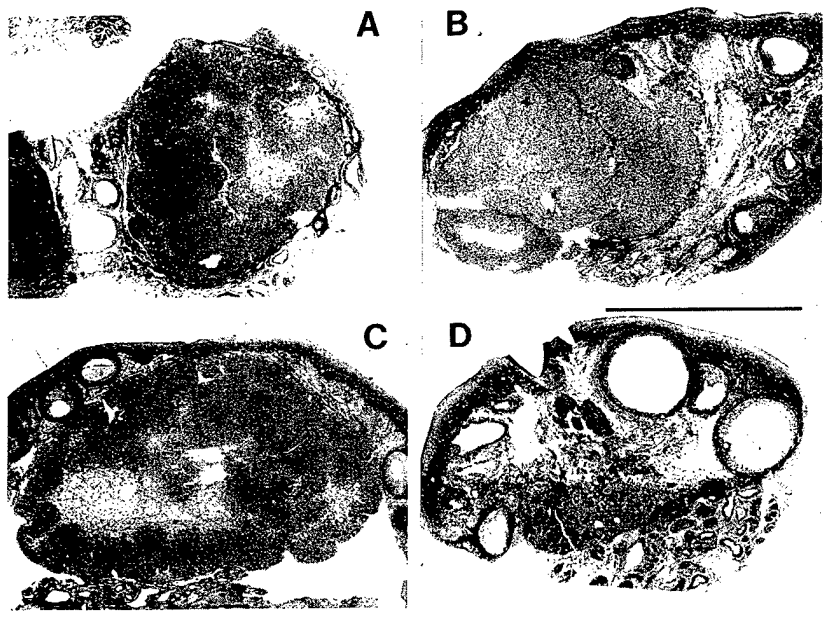

Figure 5. Light micrographs of glycolmethacrylate, haematoxylin-stained sections of a corpus luteum from a representative rhesus monkey at the mid-luteal phase after five cycles of daily treatment with (**A**) vehicle (control), (**B**) 0.01 mg/kg ZK 137 316 or (**C**) 0.03 mg/kg ZK 137 316. The corpus luteum obtained following the 0.01 mg/kg dose shows luteal morphology typical of the control. In contrast, the corpus luteum collected after the 0.03 mg/kg dose exhibits a large central cavity surrounded by luteal cells. (**D**) Section through the ovary from an animal 7 days after the oestradiol peak during cycle 5 of daily treatment with 0.1 mg/kg ZK 137 316. Note the lack of luteal tissue and the presence of antral follicles. Original magnification of each photomicrograph is ×3.5. Bar = 5 mm.

for five consecutive cycles. Likewise, prolonged treatment for three cycles with low doses of onapristone at weekly intervals (Ishwad *et al.*, 1993) or every 3 days (Katkam *et al.*, 1995) in bonnet monkeys, as well as daily mifepristone administration to women (Gemzell-Danielsson *et al.*, 1997), did not disrupt menstrual cyclicity. However, longer treatment intervals in women (Croxatto *et al.*, 1997) and non-human primates (Katkam *et al.*, 1995) can result in ovulation suppression and the cessation of regular cyclicity.

Although our daily sampling interval precludes the recognition of subtle changes in the pattern of gonadotrophin secretion, it is clear that the 0.01 and 0.03 mg/kg doses do not alter gonadotrophin release sufficiently to prevent timely follicular development, the mid-cycle gonadotrophin surge and subsequent luteal phases. Anovulation displayed by one animal during the fifth cycle of 0.03 mg/kg treatment may have resulted from a cumulative effect of chronic exposure to this dose; confirmation will require a longer treatment interval. In contrast to our study, lengthening of the follicular phase was observed in women during 1 month of low-dose (1–2 mg/day) mifepristone administration, even though menstrual cyclicity was maintained (Batista *et al.*, 1992; Croxatto *et al.*, 1993; Cameron *et al.*, 1995). However, lowering the daily dose to 0.1 or 0.5 mg mifepristone did not alter the lengths of the follicular phase or menstrual cycle in women when administered for 3 months (Gemzell-Danielsson *et al.*, 1997).

Amenorrhoea accompanied by anovulation observed in macaques receiving 0.1 mg ZK 137 316 in our study are consistent with the dose-dependent suppression of ovulation reported with increasing doses of mifepristone or onapristone in non-human primates and women (Danforth *et al.*, 1989; Ledger *et al.*, 1992; Croxatto *et al.*, 1993; Ishwad *et al.*, 1993; Katkam *et al.*, 1995; Cameron *et al.*, 1995, 1996; Gemzell-Danielsson *et al.*, 1996, 1997). Increased concentrations of FSH observed after the peak oestradiol concentration during the fifth anovulatory treatment cycle of 0.1 mg/kg ZK 137 316 probably reflect the absence of steroid or protein (e.g. inhibin; Illingworth *et al.*, 1996) hormones from the corpus luteum, which normally inhibit hypothalamic/pituitary function via negative feedback. Serum oestradiol concentrations in the 0.1 mg/kg group remained at values typical of the follicular phase of a normal menstrual cycle, indicating that these follicles were capable of responding to FSH. Increased FSH secretion during treatment with 0.1 mg/kg ZK 137 316 may contribute to the persistence of large antral follicles and the formation of cystic follicular structures in monkeys, as also noted with daily doses of mifepristone in women (Croxatto *et al.*, 1993, 1997; Cameron *et al.*, 1995). The development and maintenance of oestrogen-secreting cystic follicles poses a concern with chronic antiprogestin treatment if the daily dose suppresses the gonadotrophin surge.

Large centrums were noted in about half the corpora lutea collected at the mid-luteal phase during the fifth cycle of 0.01 and 0.03 mg/kg ZK 137 316 treatment. Since progesterone, along with the mid-cycle gonadotrophin surge, is required during the peri-ovulatory interval for follicle rupture in primates (Hibbert *et al.*, 1996), it is possible that low doses of antiprogestin act in the ovarian follicle to cause 'trapped', i.e. unovulated, oocytes or subnormal luteinization. Interestingly, luteinization without concomitant follicular rupture was observed by Croxatto *et al.* (1993) during one cycle of daily low-dose mifepristone administration in women. Progesterone concentrations during the luteal phase were normal throughout the 5 month interval of 0.01 and 0.03 mg/kg ZK 137 316

Table I. The incidence of ovarian cyclicity and amenorrhoea during the first, third and fifth consecutive cycles of daily administration with vehicle or 0.01, 0.03 or 0.1 mg/kg of the antiprogestin ZK 137 316

		Control (n = 6)	0.01 (n = 8)	0.03 (n = 8)	0.1 (n = 5)
C-1[a]	Ovarian cyclicity[b]	5	7	7	2
	Not menstruating[c]	0	3	4	3
C-3	Ovarian cyclicity	5	8	7	0
	Not menstruating	1	0	4	5
C-5	Ovarian cyclicity	5	7	6	0
	Not menstruating	1	2	4	5

[a]Treatment cycles 1, 3 and 5 (C-1, C-3 and C-5, respectively).
[b]Number of animals exhibiting ovarian cyclicity based on serum hormone concentrations typical of normal menstrual cycles in untreated macaques. One animal in the 0.01 mg/kg group did not display ovarian cyclicity during treatment cycles 1 and 5, and had a blunted mid-cycle gonadotrophin surge. One animal in the 0.03 mg/kg group had a rectal prolapse and was acyclic throughout the entire treatment interval. An additional animal receiving 0.03 mg/kg displayed an abnormally long follicular phase (~24 days) during treatment cycle 5 without a mid-cycle gonadotrophin surge.
[c]Number of animals that did not exhibit overt menstruation at the end of the treatment cycle.

Table II. Duration (days) of the follicular and luteal phases and of the menstrual cycle in rhesus monkeys receiving daily injections of vehicle or 0.01, 0.03 or 0.1 mg/kg of the antiprogestin ZK 137 316

Treatment[a]	Follicular phase			Luteal phase			Menstrual cycle		
	C-1[b]	C-3	C-5[c]	C-1	C-3	C-5[c]	C-1	C-3	C-5[c]
Control	13 ± 1	14 ± 1	13 ± 1	13 ± 2	15 ± 1	17	26 ± 1	28 ± 1	28
0.01 mg/kg	13 ± 1	14 ± 2	14 ± 2	14 ± 1	14 ± 1	15	27 ± 2	28 ± 2	27
0.03 mg/kg	12 ± 1	13 ± 1	15 ± 2	16 ± 0	15 ± 1	16	28 ± 0	28 ± 1	30
0.1 mg/kg	11[d]	NA[e]	NA	14[d]	NA	NA	25[d]	NA	NA

[a]Data represent the mean ± SEM of the number of animals that displayed ovarian activity (see Table I).
[b]Treatment cycles 1, 3 and 5 (C-1, C-3 and C-5, respectively).
[c]Represents two remaining ovarian-intact animals per group.
[d]Represents two animals displaying ovarian activity.
[e]NA = both ovarian activity and menstruation were absent.

treatment, suggesting that functional luteinization with regard to steroidogenesis occurred.

Since the 0.03 mg/kg dose of ZK 137 316 allowed ovarian cyclicity throughout the treatment interval, the cessation of menstruation by the third treatment cycle was not due to anovulation as observed in the 0.1 mg/kg group but to antagonism of progesterone action on the endometrium. Slayden *et al.* (1998) demonstrated a dose-dependent atrophy of the endometrium but not the oviducts, marked by an overall thinning of the endometrium, reduced mitotic activity in the glands, compaction of the stroma, degradation of spiral arteries and dilation of veins following five consecutive cycles of 0.03 and 0.1 mg/kg ZK 137 316 treatment. These data indicate that in rhesus monkeys, as in women (Batista *et al.*, 1992; Cameron *et al.*, 1996; Gemzell-Danielsson *et al.*, 1996, 1997) and laboratory animals (Chwalisz *et al.*, 1997), the endometrium is more sensitive than the hypothalamo–pituitary axis to the antagonistic actions of antiprogestin. Profound inhibition of endometrial growth, even in the presence of oestradiol concentrations that can elicit endometrial proliferation, is a distinct feature of continual antiprogestin treatment in non-human primates and women (van Uem *et al.*, 1989; Wolf *et al.*, 1989; Kettel *et al.*, 1992; Slayden and Brenner, 1993; Ishwad *et al.*, 1993; Slayden *et al.*, 1994; Cameron *et al.*, 1995, 1996; Katkam *et al.*, 1995; Murphy *et al.*, 1995; Gemzell-Danielsson *et al.*, 1996, 1997; Ghosh *et al.*, 1996).

In summary, dose-dependent effects of chronic antiprogestin treatment were observed in macaques that correlate with differing sensitivities of progesterone target tissues to antiprogestin action in the reproductive system. Low daily doses, i.e. 0.01 mg/kg ZK 137 316, retard endometrial growth while permitting normal ovarian/menstrual cyclicity in rhesus monkeys. Moderate doses (0.03 mg/kg) of antiprogestin exhibit greater potency at the endometrial level, which disrupts menstruation in half the animals but allows ovarian cyclicity even in the absence of menstruation. It remains to be determined whether moderate doses (i) completely suppress menstruation or cause 'silent' menstruation observable only at the endometrial level; and (ii) also prevent rupture of the preovulatory follicle without altering luteinization of the follicle wall. Higher doses of ZK 137 316 (0.1 mg/kg) act primarily to antagonize hypothalamic/pituitary function which prevents ovulation as well as ovarian cyclicity and results in amenorrhoea. Thus, a chronic low-dose regimen of the antiprogestin ZK 137 316 which permits normal ovarian/menstrual cyclicity can be considered as a potential contraceptive in women. Although the feasibility of a low-dose onapristone regimen to prevent pregnancy was confirmed recently in bonnet monkeys during chronic (4–7 months) treatment (Katkam *et al.*, 1995), ovarian cyclicity was consistently suppressed after 3 months of treatment. Our laboratory is currently evaluating the contraceptive efficacy of daily treatment with low doses of ZK 137 316 that maintain ovarian cyclicity during prolonged treatment in rhesus monkeys.

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A chronic, low-dose regimen of the antiprogestin ZK 137 316 prevents pregnancy in rhesus monkeys

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Continual administration of low doses of the antiprogestin ZK 137 316 was previously reported to permit ovarian/menstrual cyclicity, but disrupt endometrial growth in macaques. The contraceptive efficacy of this regimen was tested in female rhesus monkeys (10 per group) treated daily with vehicle (controls), 0.01 or 0.03 mg ZK 137 316 per kg body weight for 30 days before and during continual co-habitation with males of proven fertility. Treatment continued until confirmation of pregnancy or for 5 months after pair-housing with males. Mating and vaginal sperm were evident in all females. A cumulative pregnancy rate of 90% (9/10) was observed in the controls. Of the 10 animals receiving 0.01 mg/kg, four conceived during the first 2 months of pairing ($P = 0.06$) with no further conceptions. No pregnancies were observed in the 0.03 mg/kg group ($P < 0.01$). Timely, overt menses occurred at a higher frequency in the 0.01 mg/kg group than the 0.03 mg/kg group. However, corpora lutea were present in ovaries from both groups during the last treatment cycle, indicating that ovarian cycles occurred. Thus, chronic administration of low-dose ZK 137 316 that permits continued ovarian cyclicity and a high incidence of timely menses, prevents pregnancy in non-human primates. This regimen may provide a novel method of contraception for women.

Key words: antiprogestin/contraception/menstrual cycle/pregnancy/ZK 137 316

Introduction

Chronic treatment with low doses of antiprogestin may comprise a novel regimen for regulating fertility in women. Very low doses (e.g. 0.1–1.0 mg/kg) of antiprogestins administered daily throughout the menstrual cycle inhibit growth and retard maturation of the human (Batista *et al.*, 1992; Kettel *et al.*, 1992; Croxatto *et al.*, 1993; Cameron *et al.*, 1995, 1996; Gemzell Danielsson *et al.*, 1996) or non-human primate (Iswhad *et al.*, 1993; Katkam *et al.*, 1995) endometrium without causing premature menstruation. In the majority of these studies, ovarian cyclicity was maintained for up to three consecutive months of low-dose, antiprogestin treatment; however, longer treatment intervals in women (Croxatto *et al.*, 1993, 1997) and non-human primates (Katkam *et al.*, 1995) were associated with ovulation suppression and cessation of regular cyclicity. In contrast to acute administration of a large dose of antiprogestin during the follicular or luteal phase of the menstrual cycle (reviewed by Van Look and von Hertzen, 1995; Spitz *et al.*, 1996), continuous exposure to low doses of antiprogestin that maintain ovarian cyclicity avoids the need for critical timing of antiprogestin treatment in the subsequent cycle, as well as the need for accurate determination of the luteinizing hormone (LH) surge.

We recently determined a range of very low doses (0.01–0.03 mg/kg) of a new antiprogestin, ZK 137 316, which maintains ovarian and menstrual cyclicity when administered daily for five consecutive months in rhesus monkeys (Zelinski-Wooten *et al.*, 1998). This antiprogestin regimen caused a dose-dependent atrophy of the endometrium, but not the oviducts, marked by an overall thinning of the endometrium, reduced mitotic activity in the glands, compaction of the stroma, and degenerative changes in the spiral arteries and veins (Slayden *et al.*, 1998). Because implantation is unlikely to occur in such a profoundly suppressed endometrium, continuous treatment with low-dose ZK 137 316 may comprise a novel approach to contraception. Therefore, the present study was conducted to test whether chronic, low-dose regimens of ZK 137 316 have contraceptive efficacy in a non-human primate model.

Materials and methods

Animals and treatments

Adult, female rhesus monkeys ($n = 30$), exhibiting normal body weights (4.5–6.0 kg) and regular menstrual cycles of about 28 days, were kept under controlled conditions of temperature (22°C) and a standard daily light–dark cycle (12 h light:12 h dark). Each female had previously given birth to one or more live, normal, singleton infants during her reproductive years prior to this study. Individual females were placed adjacent to a male of proven fertility in cages designed to house a breeding pair. A Plexiglas barrier was positioned to separate physically the female from the male while allowing visual

and olfactory exposure. After a month of acclimatization to the caging system, females were assigned randomly (n = 10 per group) to receive at 0830–0900 hours, a single, i.m. injection of vehicle (controls, 25% ethanol/37.5% propylene glycol/37.5% saline, v/v/v, 0.5 ml; Sigma, St Louis, MI, USA), 0.01 mg or 0.03 mg ZK 137 316 per kg body weight for one month prior to (barrier in place) and up to five consecutive months during continual co-habitation with males (barrier removed).

Monkeys in each group were checked daily for menstruation during all cycles, and the duration of menstruation was recorded. Mating between pairs was confirmed by visual observation and evidence of sperm after vaginal swabs. Transabdominal ultrasonography (Ultramark 2 system with 7.5 mHz scanhead; ATL, Bothell, Washington, USA) was performed on anaesthetized females when pregnancy was suspected based on menstrual records. Fetal measurements (greatest length, biparietal diameter, femur length) were used to determine the dates of conception and delivery (Tarantal and Hendrickx, 1988). Fetal viability was determined by the presence of fetal heartbeat. Treatment continued until confirmation of pregnancy in controls, or for at least 50 days of gestation in antiprogestin-treated females. Upon pregnancy confirmation, males were separated from females, and pregnancies were allowed to progress until term. Non-pregnant animals continued to be treated for five consecutive months after pairing with males.

Body weights of each animal were obtained monthly. Regardless of gestational status, a blood sample from the saphenous vein was collected from each animal on the same day during the last month of the study. In addition, blood samples were obtained from 10 females exhibiting normal ovarian/menstrual cycles randomly selected from our colony. Complete blood counts and serum biochemistry tests (including electrolytes, glucose, lipids, proteins, enzymes and metabolic by-products) were performed by the Clinical Pathology Laboratory at Oregon regional Primate Research Center (ORPRC) and Quest Diagnostics Incorporated (Portland, OR, USA), respectively.

Reproductive behaviour

At 0800 hours each morning during the first and last month of pairing, the presence of ejaculate under the cage, as well as mounting (which included mounts with thrusting and intromission) and mating (mounting with intromission and ejaculation) behaviours were noted. The frequency of these reproductive behaviours was recorded for each pair. Of the 10 pairs in each group, the number of pairs exhibiting these behaviours were compared within a group between months, as well as between groups within a month.

Ovarian and reproductive tract morphology

Autopsies were performed on non-pregnant animals during a one-week interval in the sixth cycle of treatment with 0.01 or 0.03 mg/kg ZK 137 316 (n = 3 per group) for evaluation of pathological changes in tissues. As part of an ongoing study, reproductive tracts were also removed from non-pregnant animals at autopsy in the sixth cycle of treatment with 0.01 or 0.03 mg/kg ZK 137 316 (n = 3 per group). Toxicological studies were performed on these additional reproductive tissues during the final treatment interval in the present study. This allowed a further opportunity to (i) extend and confirm previous observations (based on three animals per group), (ii) confirm the presence of a corpus luteum regardless of whether menstruation was evident, and (iii) look for silent menstruation which would not be evident from a vaginal swab, particularly in the 0.03 mg/kg group. In animals exhibiting normal menstrual cycles (n = 2, 0.01 mg/kg and n = 1, 0.03 mg/kg), autopsies occurred at midluteal phase (~19–21 days after the onset of menses). The reproductive tract from the one vehicle-treated animal that did not become pregnant was removed surgically at midluteal phase for comparison with the non-pregnant, antiprogestin-treated animals. Tissues were dissected and prepared for histological analyses as previously described (Slayden *et al.*, 1997). Briefly, tissue samples were fixed in 2% glutaraldehyde and 3% paraformaldehyde, embedded in glycol methacrylate, sectioned and stained.

Statistical analyses

The number of female–male pairs that exhibited mounting and mating was compared within and between groups using Fisher's exact test (when the numerator was <5) or χ^2 analyses. Pregnancy rates between control and antiprogestin-treated groups were compared by Fisher's exact tests. Blood parameters and body weights of non-pregnant or pregnant animals within the month of treatment were analysed by one-way analysis of variance, followed by Neuman–Kuehls test for comparisons among groups. One-way analysis of variance with one repeated measure was used to analyse body weights of non-pregnant females within a group prior to and during treatment, with comparisons among months made using Neuman–Kuehls test. Comparisons of blood parameters in animals receiving 0.03 mg/kg during and 2 months after treatment as well as between the 0.03 mg/kg group and untreated females exhibiting normal menstrual cycles were made using paired and unpaired t-tests, respectively.

Results

Although a detailed behavioural study (i.e. focal sampling) was not conducted, mounting with thrusting and intromission as well as mating was noted at least once in both vehicle- and antiprogestin-treated animals during daily morning observations in the first and last month of pairing (data not shown). There were no differences between the vehicle- and antiprogestin-treated groups with regard to the number of pairs that exhibited mounting or mating in either the first or final month of pairing. Most importantly, evidence of spermatozoa after vaginal swabbing confirmed that mating occurred between each of the 30 pairs throughout the interval of co-habitation (treatment cycles 2 to 5).

Table I depicts the number of animals that conceived during each month of daily treatment with vehicle (controls), 0.01 or 0.03 mg ZK 137 136/kg body weight (n = 10 per group). In the control group, pregnancies were noted beginning in the first paired cycle (October), with the majority occurring in the first two months of pairing. After five consecutive months of paired housing, a cumulative pregnancy rate of 90% (9/10) was observed in the controls. In contrast, only 4/10 females receiving the 0.01 mg/kg dose became pregnant (P = 0.06 relative to controls), with conceptions occurring only within the first two months of paired housing. No pregnancies were observed in the 0.03 mg/kg group (P < 0.01 relative to controls and 0.01 mg/kg groups) throughout the interval of co-habitation.

Table II outlines the outcome of pregnancies in animals that conceived during treatment with vehicle or 0.01 mg ZK 137 316/kg body weight. In the control group, 8/9 pregnancies resulted in live, singleton births (five males, three females); the remaining pregnant animal miscarried at gestational day 67. Each of the four pregnant females in the 0.01 mg/kg group had a normal delivery of a single live infant (two males, two females). The mothers of the two females received

Table I. Numbers of female rhesus monkeys conceiving during daily administration of vehicle (control), 0.01 or 0.03 mg ZK 137 316/kg body weight for six consecutive cycles during paired housing (1 female:1 male)

Treatment (n = 10)	Treatment cycle						Pregnancy rate (%)
	1 September Barrier	2 October Paired	3 November	4 December	5 January	6 February	
Control	–	3	3	2	1	0	90[a]
0.01 mg/kg	–	2	2	0	0	0	40[a]
0.03 mg/kg	–	0	0	0	0	0	0[b]

During treatment cycle 1 (September), the barriers between females and males were in place. Co-habitation was initiated at the onset of treatment cycle 2 (October; first paired cycle) and continued through treatment cycle 6 (February; fifth paired cycle).
[a,b] Values with different superscripts within column are significantly different ($P < 0.05$).

Table II. Pregnancy outcome of animals that conceived during treatment with vehicle (controls) or 0.01 mg ZK 137 316/kg body weight

Treatment group	Gestation length (days)	Number of live births	Infant weight (g)	
			Males[a]	Females[a]
Control	165 ± 1	8/9[b]	484 ± 19 (5)	463 ± 14 (3)
0.01 mg/kg	165 ± 5	4/4	480, 575 (2)	420, 442 (2)

[a]Number in parentheses indicates the number of males or females born.
[b]One animal miscarried at 67 days of gestation.

0.01 mg/kg ZK 137 316 for 50 days of gestation, and those of the two males were treated for 82 and 86 days of gestation. There was no difference in the length of gestation between the control and the 0.01 mg/kg groups (Table II). Delivery dates estimated from fetal measurements taken at ultrasonography were within 5 days of the actual delivery date, except in two cases where the estimated delivery dates were within 10 and 14 days. Birthweights of all infants in both groups (Table II) are within the normal range for male and female rhesus infants at ORPRC. All infants appeared normal at birth and to date have not exhibited any physical abnormalities.

There were no differences in body weights among groups during the pretreatment interval and the first month of treatment as well as throughout treatment with antiprogestin in all non-pregnant animals (data not shown). Pregnant animals in the control and 0.01 mg/kg groups typically began to gain weight between the first and second trimester.

Table III summarizes the number of non-pregnant animals exhibiting menstruation during the pretreatment and treatment (six consecutive months) intervals. There were no differences in the length of menstrual cycles or duration of menses among animals in each group both prior to and, in non-pregnant animals exhibiting overt menses, during treatment (data not shown). Whereas monkeys typically menstruated at the end of the pretreatment cycle, only 16 of 30 animals menstruated at the end of treatment cycle 1. Since this was apparent in all groups, those monkeys not menstruating were likely undergoing stress related to moving to a new environment, including proximity to a male (prior to barrier removal). All six of the remaining non-pregnant animals in the 0.01 mg/kg group, but only 4/10 in the 0.03 mg/kg group, typically displayed overt menstruation at monthly intervals throughout treatment.

Ovariectomy at midluteal phase of the last treatment cycle in animals of all groups revealed a single corpus luteum on one ovary, and numerous, small (~0.5 mm diameter) follicles on the contralateral ovary, regardless of whether normal menstrual cyclicity was evident (data not shown). No cystic structures were observed. Five of the six antiprogestin-treated animals had a corpus luteum morphologically similar to and serum progesterone concentrations (1.07–6.42 ng/ml) typical of normally cycling macaques at midluteal phase. The remaining animal in the 0.03 mg/kg group exhibited a comparatively smaller corpus luteum, a serum progesterone concentration of 0.10 ng/ml, and evidence of endometrial menstruation indicative of late luteal phase, although overt menstruation was not observed.

Morphology of the endometrium and oviducts following vehicle, 0.01 or 0.03 mg/kg treatments was identical to that observed in our previous study (Slayden *et al.*, 1997), thus data from the current study are not shown. Relative to vehicle-treated animals, a dose-dependent decrease in endometrial thickness associated with compaction of the endometrial stroma was observed in animals treated with 0.01 or 0.03 mg/kg antiprogestin. In controls and the 0.01 mg/kg group, the epithelium of the oviducts was deciliated and non-secretory, typical of midluteal phase. In contrast, increased oviductal ciliation was noted in the 0.03 mg/kg group.

Table IV lists salient values from serum biochemistry and haematological determinations in untreated females exhibiting normal menstrual cycles selected randomly from the colony, as well as from non-pregnant animals during the final cycle of treatment with vehicle, 0.01 or 0.03 mg ZK 137 316/kg and two months after the final injection of the 0.03 mg/kg dose. General indices of circulating glucose and ions, renal function (blood urea nitrogen, creatinine), liver function (e.g. total

Table III. Numbers of non-pregnant monkeys exhibiting menstruation during the pretreatment interval and daily administration of vehicle (control), 0.01 or 0.03 mg ZK 137 316/kg body weight for six consecutive cycles

Treatment (n = 10)[a]	Pretreatment August	Treatment cycle[a] 1 September Barrier	2 October Paired	3 November	4 December	5 January	6 February
Control							
Non-pregnant	10	10	7	4	2	1	1
Menstruating	9	4	5	4	2	1	1
0.01 mg/kg							
Non-pregnant	10	10	8	6	6	6	6
Menstruating	8	7	6	6	3	6	3
0.03 mg/kg							
Non-pregnant	10	10	10	10	10	10	10
Menstruating	7	5	4	4	3	4	1

[a]The number of non-pregnant animals/group declines over the treatment interval as animals conceived during paired housing with males.

Table IV. Concentrations of blood constituents in female rhesus monkeys during the sixth cycle of treatment with vehicle (controls), 0.01 or 0.03 mg/kg ZK 137 316

Blood constituent[a]	Control[b] Non-pregnant (n = 1)	Untreated, Normal cycles[b] (n = 10)	Treatment group (mg/kg) 0.01 Non-pregnant (n = 6)	0.03 Non-pregnant (n = 10)	0.03 Post-treatment (n = 7)
Glucose (mg/dl)	64	64 ± 2	62 ± 1	61 ± 3	75 ± 4
BUN[c] (mg/dl)	17	20 ± 1	17 ± 0	19 ± 1	20 ± 1
Creatinine (mg/dl)	0.8	0.8 ± 0.1	0.7 ± 0.0	0.8 ± 0.0	0.8 ± 0.0
Sodium (mEq/l)	151	150 ± 1	148 ± 0	149 ± 0	147 ± 0
Potassium (mEq/l)	3.9	4.3 ± 0.1	3.8 ± 0.1	4.1 ± 0.1	3.9 ± 0.1
Chloride (mEq/l)	111	109 ± 1	111 ± 1	110 ± 1	108 ± 0
Total protein (g/dl)	7.5	7.9 ± 0.2	7.4 ± 0.1	7.5 ± 0.2	7.7 ± 0.1
Albumin (g/dl)	3.9	4.1 ± 0.1	3.9 ± 0.0	3.8 ± 0.1	4.1 ± 0.2
Globulin (g/dl)	3.6	3.8 ± 0.1	3.6 ± 0.1	3.7 ± 0.2	3.6 ± 0.2
Albumin/globulin	1.1	1.1 ± 0.1	1.1 ± 0.0	1.1 ± 0.1	1.2 ± 0.1
GGT[d] (U/l)	59	61 ± 4	60 ± 7	57 ± 2	71 ± 3
Alkaline phosphatase (U/l)	86	54 ± 18	135 ± 19	116 ± 15	143 ± 18
Lactic dehydrogenase (U/l)	188	352 ± 40	174 ± 6	227 ± 24	253 ± 41
AST[e] (U/l)	25	28 ± 1	23 ± 2	26 ± 2	22 ± 1
ALT[f] (U/l)	34	35 ± 7	41 ± 4	40 ± 3	41 ± 8
Leukocytes ($\times 10^3$/mm^3)	7.1	9.3 ± 0.9	4.5 ± 0.3	7.6 ± 0.8	8.5 ± 1.5
Erythrocytes ($\times 10^6$/mm^3)	5	6.2 ± 0.1	5.0 ± 0.2	5.3 ± 0.1	5.4 ± 0.2
Neutrophils (%)	28	48 ± 3	42 ± 5	56 ± 4	54 ± 6
Lymphocytes (%)	64	50 ± 3	56 ± 5	40 ± 4	46 ± 6
Packed cell volume (%)	36	43.1 ± 1.0	35.5 ± 0.7	37.2 ± 0.7	7.8 ± 1.0
Haemoglobin (g/dl)	11.7	13.9 ± 0.3	11.8 ± 0.2	12.2 ± 0.2	12.4 ± 0.3

[a]Values represent mean ± SEM for animals in each group.
[b]Represents 10 randomly selected females in the colony exhibiting normal menstrual cycles.
[c]Blood urea nitrogen.
[d]γ-Glutamyl transferase.
[e]Aspartate aminotransferase.
[f]Alanine aminotransferase.

protein, albumin/globulin ratio, γ-glutamyl transferase, alanine aminotransferase), muscle function (e.g. lactate dehydrogenase, aspartate aminotransferase) and blood cell constituents (e.g. erythrocytes) or any other parameters typically measured revealed no significant difference between untreated females and non-pregnant females treated with either the 0.01 or 0.03 mg/kg dose. Likewise, concentrations obtained from the 0.03 mg/kg group during the final treatment cycle were similar to post-treatment concentrations in these same animals (Table IV). Although comparisons were not made between pregnant females in the control and 0.01 mg/kg groups due to differences in gestational stage at sampling, their values were typical of pregnant females in the colony (data not shown).

Discussion

This is the first study to demonstrate that a chronic regimen of low-dose ZK 137 316 which permits continued ovarian/

menstrual cyclicity (Zelinski-Wooten *et al.*, 1998) can prevent pregnancy in a non-human primate model. Dramatic antiproliferative effects were observed in the endometrium of non-human primates (Ishwad *et al.*, 1993; Katkam *et al.*, 1995; Slayden *et al.*, 1998) during chronic administration of low-dose antiprogestin. Although there are no comparable studies in women, the available data from short-term studies with low doses of RU 486 (Batista *et al.*, 1992; Kettel *et al.*, 1992; Croxatto *et al.*, 1993; Cameron *et al.*, 1996; Gemzell Danielsson *et al.*, 1996, 1997a) indicate that the endometrium was retarded, and there was no evidence for unopposed oestrogen effects. Collectively, these studies support the concept that this regimen could possibly confer protection from pregnancy by preventing implantation, but proof that changes in the endometrium were incompatible with pregnancy was needed. Daily treatment of females with 0.03 mg ZK 137 316/kg body weight was an effective contraceptive throughout the 5-month interval of pairing with males, in spite of continued ovulatory cycles (Zelinski-Wooten *et al.*, 1998). Although the 0.01 mg/kg dose was contraceptive in the majority of animals, it was initially less effective than the 0.03 mg/kg dose. The feasibility of low-dose regimens of onapristone to prevent pregnancy was demonstrated in bonnet monkeys (Ishwad *et al.*, 1993; Katkam *et al.*, 1995); however, ovarian cyclicity was suppressed with prolonged treatment. The efficacy of chronic, low-dose ZK 137 316 as a contraceptive in the non-human primate model provides critical evidence that this novel regimen, which maintains ovarian cyclicity at both doses as well as menstrual cyclicity in a dose-dependent manner, can be considered as a potential contraceptive in women.

Chronic exposure to 0.01 or 0.03 mg ZK 137 316/kg body weight was well tolerated by female macaques, with no detrimental effects of this regimen noted in serum biochemical and haematological determinations. Ongoing studies of the toxicology and pathology of this regimen will be published elsewhere, but preliminary findings have raised no serious concerns. The use of ZK 137 316 at very low doses may preclude detrimental long-term effects. Endometrial hyperplasia seems unlikely in view of the extensive inhibition of endometrial growth and vascular degeneration following this antiprogestin regimen in macaques (Slayden *et al.*, 1998; this study) and in women receiving chronic, low-dose mifepristone (Batista *et al.*, 1992; Kettel *et al.*, 1992; Croxatto *et al.*, 1993; Cameron *et al.*, 1996; Gemzell Danielsson *et al.*, 1996, 1997a). However, consideration must be given to possible deleterious effects of unopposed oestrogen in tissues where classical inhibition of progesterone action in the presence of circulating oestradiol concentrations typical of the follicular phase allows manifestation of oestrogen-dependent effects. While the oviducts were devoid of histological abnormalities in this and our previous study (Slayden *et al.*, 1998), the long-term effects of this antiprogestin regimen on other oestrogen-dependent tissues such as the cervix, vagina or mammary glands in non-human primates warrant further investigation.

The antiproliferative effects of continuous, low-dose ZK 137 316 antiprogestin appear to be endometrium-specific (Slayden *et al.*, 1998; this study). Because implantation is unlikely to occur in this milieu, the endometrium may be the primary site of antiprogestin action to prevent pregnancy. Support for this concept is also provided by clinical observations of endometrial growth retardation in women receiving chronic, low-dose mifepristone (Batista *et al.*, 1992; Kettel *et al.*, 1992; Croxatto *et al.*, 1993; Cameron *et al.*, 1996; Gemzell Danielsson *et al.*, 1996, 1997a). Perhaps a reduction in endometrial secretory activity, as indicated by asynchronous expression of glycodelin (Batista *et al.*, 1992; Gemzell Danielsson *et al.*, 1997a), depressed lectin binding (Gemzell Danielsson *et al.*, 1997a) or expression of leukaemia inhibitor factor (Gemzell Danielsson *et al.*, 1997b) in endometrial glands, may contribute to the inhibition of endometrial receptivity and implantation following continuous exposure to low doses of mifepristone.

However, chronic exposure to low-dose antiprogestin at other sites in the reproductive tract (i.e. oviducts, cervix, vagina) may prevent timely gamete transport, fertilization or early embryonic development which could synergistically contribute to the contraceptive efficacy of this treatment regimen in primates. In rodents, embryo transport through the oviducts was accelerated following post-coital administration of mifepristone (Kendle and Lee, 1980; Psychoyos and Prapas, 1987; Vinijsanun and Martin, 1990) leading to reduced numbers of embryos recovered from the uterus (Roh *et al.*, 1988; Roblero and Croxatto, 1991). In-vivo administration of mifepristone (van Uem *et al.*, 1989) or a steroid synthesis inhibitor (Zelinski-Wooten *et al.*, 1994) to gonadotrophin-treated macaques did not affect oocyte nuclear maturation, but steroid depletion in macaques impaired fertilization (Zelinski-Wooten *et al.*, 1994). In contrast, normal fertilization was noted in mice that received mifepristone during mating (Roh *et al.*, 1988). Pre- and post-coital exposure of female rodents to mifepristone retarded the development of embryos to the morula or blastocyst stages (Psychoyos and Prapas, 1987; Roh *et al.*, 1988; Loutradis *et al.*, 1991; Roblero and Croxatto, 1991), but macaque preimplantation embryos developed normally after acute exposure to mifepristone for 24 h *in vitro* (Wolf *et al.*, 1990). However, a single dose of mifepristone given at the time of ovulation/fertilization in rhesus monkeys impaired preimplantation embryo viability and delayed the transition from morula to blastocyst *in vivo* (Ghosh *et al.*, 1996, 1997). Thus, progesterone-mediated functions may contribute to preimplantation embryonic development in macaques, but direct actions on primate embryos have not been defined. The ability of chronic, low-dose antiprogestin to disrupt these events in primates remains to be established.

Normal birth and post-natal development following maternal treatment with 0.01 mg/kg ZK 137 316 during the first trimester of pregnancy in rhesus monkeys suggests that this regimen does not adversely affect the progression of fetal development to term. Whether embryonic or fetal exposure *in utero* to the 0.03 mg/kg regimen has deleterious effects on subsequent development is unknown. Developmental lesions were also absent in live offspring following acute exposure of macaque embryos to high doses of mifepristone *in vitro* or *in vivo* during the immediate post-implantation period (Wolf *et al.*, 1990). While possible teratogenic effects of a chronic antiprogestin regimen in the non-human primate model cannot be

entirely ruled out, the use of relatively low doses that confer protection from pregnancy may present a low risk. Larger trials are needed to evaluate fetal well-being following maternal treatment with continuous, low-dose antiprogestin.

Timely menstruation at regular intervals occurred more frequently in animals that received the 0.01 mg/kg dose than those treated with 0.03 mg/kg, as noted in our previous study (Zelinski-Wooten *et al.*, 1998). The suppressive effects of 0.03 mg/kg ZK 137 316 on the endometrium after chronic administration were greater relative to the 0.01 mg/kg dose (Slayden *et al.*, 1998; this study), and most likely contributed to the increased prevalence of amenorrhoea in this group of animals. Nevertheless, our extensive observations that normal circulating steroid and gonadotrophin concentrations occur throughout a 5-month interval of 0.01 and 0.03 mg/kg ZK 137 316 treatment suggest that lack of overt menstruation in the 0.03 mg/kg group does not equate with cessation of ovarian cyclicity during this antiprogestin regimen (Zelinski-Wooten *et al.*, 1998). Likewise, the presence of a functional corpus luteum or, in one instance, a regressing corpus luteum accompanied by endometrial menstruation, was noted in animals in the 0.03 mg/kg group that did not display regular, overt menstruation during antiprogestin treatment. Since this antiprogestin regimen allows normal ovarian cyclicity with or without overt menstruation, a return to normal ovarian cyclicity after cessation of treatment is not a concern; however, restoration of fertility awaits confirmation.

In conclusion, a chronic regimen of low-dose ZK 137 316 that permits continued ovarian/menstrual cyclicity (Zelinski-Wooten *et al.*, 1998) prevents pregnancy without detrimental side effects in a non-human primate model. Although the 0.01 mg/kg dose was contraceptive in the majority of animals, it was initially less effective than the 0.03 mg/kg dose. Perhaps a 'step-down' regimen, i.e. sequential treatment with 0.03 mg/kg for the first 2 months followed by continued treatment with 0.01 mg/kg, would confer earlier protection from pregnancy. The cyclic maintenance of normal levels of endogenous steroids, combined with the very low doses of ZK 137 316 provides a more 'physiological' means of fertility control than acute administration of large doses of antiprogestin (or chronic treatment with oral contraceptives) that suppress ovulation. Endometrial atrophy following chronic, low-dose ZK 137 316 is unlikely to support implantation (Slayden *et al.*, 1998), but other sites of antiprogestin action may contribute to the contraceptive efficacy of this treatment regimen. Thus, this novel regimen of ZK 137 316 can be considered as a potential clinical contraceptive in women.

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OUTSTANDING CONTRIBUTION

Chronic treatment of cycling rhesus monkeys with low doses of the antiprogestin ZK 137 316: morphometric assessment of the uterus and oviduct

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The long-term effects of the antiprogestin ZK 137 316 on reproductive tract morphology in rhesus macaques were investigated. The monkeys were injected daily (i.m.) for five menstrual cycles with vehicle or 0.01, 0.03 or 0.1 mg ZK 137 316/kg body weight. Reproductive tracts (n = 3/group) were collected during the mid-luteal phase (day 8) of the fifth cycle in the control, 0.01 and 0.03 mg/kg groups, or 6–7 days after the oestradiol peak in the 0.1 mg/kg group. ZK 137 316 treatment resulted in a dose-dependent atrophy of the endometrium, marked by reduced mitotic activity in the glands, compaction of the stroma, degradation of spiral arteries and dilation of veins. There was no effect of ZK 137 316 on myometrial or oviductal weight. Treatment with 0.1 and 0.03 mg/kg, but not 0.01 mg/kg resulted in fully ciliated and secretory oviducts, indicating a dose-dependent blockade of progesterone antagonism of oestrogen-dependent oviductal differentiation. In the endometrium, the suppressive action of progesterone on oestrogen and progestin receptors was also blocked by ZK 137 316 in a dose-dependent manner. However, endometrial atrophy appeared due to inhibition of progesterone action together with a blockade of oestrogen-dependent proliferation. The profoundly suppressed endometrium produced by chronic low-dose ZK 137 316 treatment is unlikely to support implantation. Such treatment may therefore provide a novel contraceptive modality.

Key words: antiprogestin/antiproliferative/endometrium/oestrogen action/macaque

Introduction

Antiprogestins including mifepristone (RU 486), onapristone (ZK 98 299) and ZK 137 316 are structurally related, synthetic ligands for the progesterone receptor, which antagonize progesterone action. Because progesterone action is essential for normal establishment of pregnancy (Hodgen, 1985), it has long been recognized that antiprogestins are effective contragestational agents (Neef *et al.*, 1984; Baulieu, 1987). Several reports suggest that these compounds, if administered acutely at key times in the fertile cycle, may also function as contraceptives (Nieman *et al.*, 1987; Ishwad *et al.*, 1993; Katkam *et al.*, 1995).

Antiprogestins, including RU 486 (Wolf *et al.*; 1989; Slayden *et al.*, 1993; Slayden and Brenner, 1994) and onapristone (Chwalisz *et al.*, 1992), can inhibit endometrial proliferation in ovariectomized oestrogen-treated macaques. This inhibitory effect of antiprogestins in oestrogenized monkeys may be endometrium specific, because RU 486 treatment did not inhibit oestrogen stimulation of oviductal differentiation (Slayden and Brenner, 1994). Treatment of cycling macaques (Collins and Hodgen, 1986) and women (Liu *et al.*, 1987) with high doses of the antiprogestin RU 486 resulted in altered menstrual cycles and a lengthened intermenstrual interval. Continuous long-term administration of high doses of RU 486 to women resulted in acyclic ovarian function and amenorrhoea (Kettel *et al.*, 1991). These effects appear to be dose-dependent because lower doses of RU 486 delay endometrial maturation without affecting ovarian cyclicity or menstrual cycle length (Kettel *et al.*, 1991; Murphy *et al.*, 1992; Gemzell-Danielsson *et al.*, 1996). Weekly treatment of cycling bonnet monkeys with low doses of onapristone inhibited endometrial maturation during the luteal phase of the cycle without affecting folliculogenesis, ovulation or menstrual cycle length (Ishwad *et al.*, 1993). Recently, Croxatto's laboratory (Croxatto *et al.*, 1997) reported that treatment of women with 1 mg RU 486 per day for 5 months disturbed endometrial maturation. However, even this low dose of antiprogestin suppressed ovulation in 50% of test cycles. No previous studies have evaluated the endometrial effects in macaques of long-term daily administration of antiprogestin at doses which will not alter ovarian or menstrual cyclicity.

Zelinski-Wooten *et al.* (1997a) reported on the effects of various low doses of ZK 137 316 on cyclic ovarian function, as judged from the patterns and concentrations of pituitary gonadotrophic hormones and sex steroids (oestrogen and progesterone), as well as the incidence of menses. This report summarizes the effects of these treatment regimens on the morphology of the reproductive tract. Preliminary reports of our findings on ovarian/menstrual cyclicity (Zelinski-Wooten *et al.*, 1997b) and histology of the reproductive tract (Slayden *et al.*, 1997) have been published in abstract form.

Materials and methods

Animal treatments

Animal care and housing were provided by the veterinary staff of the Oregon Regional Primate Research Center, in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Sexually mature rhesus monkeys with normal menstrual cycles were injected i.m. daily for five menstrual cycles with ZK 137 316 (Schering AG, Berlin, Germany) dissolved in 37.5% Hanks' balanced salt solution, 37% 1,2-propanediol, and 25% ethanol (Zelinski-Wooten *et al.*, 1997a). Four groups were treated: vehicle only (control), 0.01, 0.03 or 0.1 mg ZK 137 316 /kg body weight. Reproductive tracts ($n = 3$ per group) were collected by laparotomy during mid-luteal phase (day 8) of the fifth cycle in the control, 0.01 and 0.03 mg/kg groups, or 6–7 days after the oestradiol peak in the 0.1 mg/kg group (Zelinski-Wooten *et al.*, 1997a).

Uteri were separated from the cervix and oviducts and quartered along the longitudinal axis. Cross-sections (2 mm thick) from two uterine quarters were cut freehand with a razor blade and prepared for immunocytochemistry (ICC) and morphological study. Endometrial and myometrial weights were obtained from the remaining two quarters after the endometrium was separated from the myometrium with fine scissors. The oviducts were dissected free from fat and connective tissue and weighed. Samples of fimbriae and ampulla were prepared for morphological study.

Histology and immunocytochemistry

For histology, tissue samples were fixed in 2% glutaraldehyde and 3% paraformaldehyde, embedded in glycol methacrylate (GMA), sectioned (uterus, 2 μm; oviduct 1.5 μm) and stained with Gill's Hematoxylin (Sandow *et al.*, 1979).

For ICC of oestrogen receptor (ER), progesterone receptor (PR) and Ki-67, tissues were prepared as recently described (Slayden *et al.*, 1993). Samples of fresh tissue were microwave stabilized (Slayden *et al.*, 1995) in an Amana Radarrange Touchmatic microwave oven (Amana, IA, USA) for 7 s in 0.5 ml Hanks' balanced salt solution (Gibco, Gaithersberg, MD, USA), then chilled on ice in 10% sucrose dissolved in 0.1 M phosphate buffered saline (PBS; Sigma, St Louis, MO, USA), mounted in Tissue Tek II OCT (Miles Inc. Elkhart, IN, USA) and frozen in liquid propane. Cryostat sections (5 μm) were thaw-mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburg, PA, USA), placed on ice at 4°C, and microwaved for 2 s. Microwave-treated sections were fixed (0.2% picric acid, 2% paraformaldehyde in PBS) for 10 min and thoroughly rinsed. Slides were treated for 20 min with a non-specific goat serum, then incubated overnight at 4°C with monoclonal anti-ER (1D-5; Biogenex, San Ramon CA), anti-PR (JZB-39; provided by Geoffrey Greene, University of Chicago, Chicago, USA) or anti-Ki-67 antigen (Dako Corp., Carpinteria, CA, USA). The primary antibody was reacted with either biotinylated anti-mouse IgG (for 1D-5 and anti Ki-67) or anti-rat IgG (for JZB-39) second antibody and detected with an avidin–biotin peroxidase kit (Vector Laboratories, Burlinghame, CA, USA).

Photomicrography

Low power photographs were made with an Olympus OM-system 38 mm macro lens and Technical Pan film (Eastman Kodak, Rochester, NY, USA). Low power prints were digitized with a Hewlett Packard Scanjet 4c/T. High power digital images were made with Zeiss planapochromatic lenses and captured through a MTI CCD 72 digital camera (Dage Corp., Michigan City, ID, USA) with the Optimas 3.0 (Optimas Inc, Seattle, WA, USA) image analysis software package. Digital images were adjusted for sharpness and contrast with Adobe Photoshop (Adobe Systems, Seattle, WA, USA) and photomicrographs were printed on a Sony Mavigraph dye sublimation printer (Sony Corp., Tokyo, Japan).

Morphometrics

The abundance of oviductal ciliated cells, endometrial mitotic cells and endometrial Ki-67-positive cells was determined by a trained observer who used an ocular micrometer grid to define microscope fields and counted between 1200 and 5000 cells per animal with the aid of a mechanical tabulator. The abundance of oviductal ciliated cells, and Ki-67-positive endometrial nuclei was expressed as a percentage of total epithelial cells. The mitotic index represented the number of mitotic cells per 1000 epithelial cells. Endometrial stromal cell density values were determined with the Optimas 3.0 image analysis software package. For this analysis, 10 non-overlapping fields of endometrial stroma (30 000 μm^2 each) in the upper functionalis of each specimen were analysed. The number of stromal cell nuclei per unit area (standardized to 10 000 μm^2) provided an index which reflected the degree to which the endometrial stroma became expanded (more oedematous) or compacted (less oedematous).

Tissue weights, the percentage of fimbrial ciliation, the abundance of mitotic cells, the percentage of Ki-67-positive uterine epithelial cells and stromal compaction were statistically compared by analysis of variance. Significant differences among means were determined by Fisher's protected least significant difference test (Petersen, 1985).

Results

Ovarian/menstrual cyclicity

All monkeys in the control and 0.01 mg/kg groups displayed ovarian cycles with follicular and luteal phases of normal length and typical patterns and concentrations of serum oestradiol and progesterone, as well as mid-cycle surges of gonadotrophins (Zelinski-Wooten *et al.*, 1997a). Likewise, these animals displayed timely menses at the end of cycles preceding hysterosalpingectomy. Although monkeys treated with 0.03 mg/kg typically displayed ovarian cycles with the expected hormonal patterns, approximately half of these animals did not mense at the end of the cycle (Zelinski-Wooten *et al.*, 1997a). Two of three animals in the 0.03 mg/kg group whose reproductive tract was removed did not mense at the end of the cycle preceding surgery. In contrast, monkeys treated with 0.1 mg/kg did not exhibit normal menstrual cycles as early as the first treatment cycle. Thereafter, oestradiol concentrations rose to mid-follicular phase concentrations but failed to reach concentrations typical of the preovulatory peak, gonadotrophin surges were absent, and circulating progesterone remained at baseline concentrations (Zelinski-Wooten *et al.*, 1997a). All monkeys in the 0.1 mg/kg group were amenorrhoeic for at least 3 months prior to removal of the reproductive tract.

Endometrial morphology

The endometrium of the vehicle-injected controls was in a hypertrophied, progestational (secretory) state consistent with the effects of progesterone during the mid-luteal phase (Figure 1a). The glands were sacculated, the stroma was expanded in both the functionalis and basalis zones (Figures 2a and e), and the spiral arteries were hypertrophied (Figure 2i). In the basalis zone, the glandular epithelium was tall columnar and mitotically active (Figure 2e) and small veins were abundant (Figure 2m).

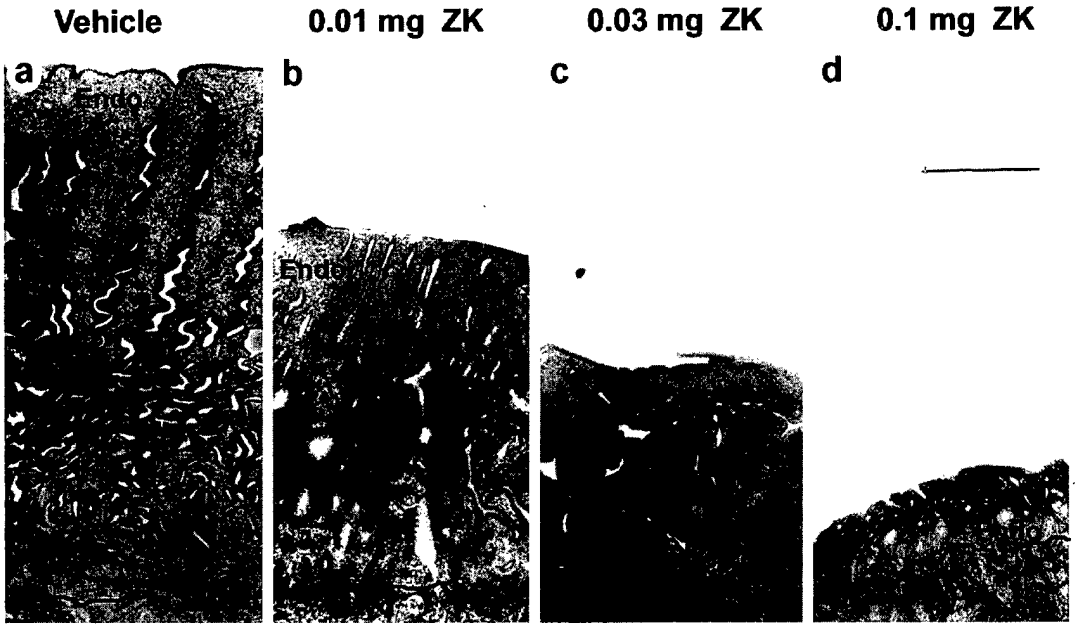

Figure 1. Low power micrographs of GMA-embedded, haematoxylin-stained sections of endometrium from vehicle and ZK 137 316-treated monkeys. All figures are at the same magnification. Bar, 1 mm. (**a**; vehicle controls) The endometrium (Endo) displayed a hypertrophied state marked by stromal expansion and the beginning of glandular sacculation. (**b**; 0.01 mg/kg ZK 137 316) The endometrium was reduced in thickness with tubular glands and a more compacted stroma. (**c**; 0.03 mg/kg ZK 137 316) The endometrium showed a striking reduction in thickness, the abundance of glands was noticeably reduced. (**d**; 0.1 mg/kg ZK 137 316) Thickness of the endometrium was further reduced, and the glands were almost absent. Myometrium is indicated (Myo). Bar, 1 mm.

Treatment with ZK 137 316 at all doses inhibited progestational differentiation of the endometrium. This effect was associated with an increase in stromal compaction and a decrease in number and sacculation of endometrial glands. Compared to vehicle-injected controls, daily treatment with ZK 137 316 resulted in an overall thinning of the endometrium (compare Figure 1a–d), which was maximal in the 0.03 and 0.1 mg/kg groups. Thinning of the endometrium was associated with a significant reduction of endometrial wet weight (Table I; $P < 0.05$), but no effect on myometrial wet weight was detected. We calculated the endometrial/myometrial wet weight ratio to adjust for overall reproductive tract size within each treatment group (Table I). Analysis of this weight ratio revealed a significant difference between each of the treatment groups ($P < 0.05$), suggesting that the ZK 137 316-induced reduction in endometrial mass was dose-dependent.

At the lowest dose of ZK 137 316 tested (0.01 mg/kg), the glandular epithelium of the functionalis zone appeared proliferative, but was reduced in height from columnar to low cuboidal. In the basalis zone, the epithelium was also cuboidal, but mitotic cells were abundant. Treatment with higher doses of ZK 137 316 (0.03 and 0.1 mg/kg) resulted in further atrophy of the glandular epithelial cells in both the functionalis and basalis zones (see Figures 2a–d and 2e–f).

Treatment with ZK 137 316 at all doses caused hyalinization of the walls of the spiral arteries with replacement of the tunica media and flattening of the endothelium, suggestive of vascular degeneration (compare Figure 2i–l). These vessels were almost completely absent in the 0.1 mg/kg group. Compared with vehicle-treated controls, ZK 137 316 treatment at all doses also produced venous dilation and increased hyalinization of the venous walls (compare Figures 2m–p).

Endometrial cell proliferation

The effects of ZK 137 316 treatment on Ki-67 antigen staining in the endometrium is shown in Figure 3 and quantitative data on both the percentage of Ki-67 positive cells and the mitotic index (number of mitotic figures/1000 epithelial cells) are presented in Table I.

In the functionalis zone from vehicle-treated controls, mitotically active epithelial cells were undetectable and few Ki-67 positive cells were evident (Figure 3a), consistent with the suppressive effects of progesterone on oestradiol action during the luteal phase of the menstrual cycle in this zone. In the functionalis, treatment with ZK 137 316 resulted in a dose-dependent increase in the percentage of Ki-67 positive cells (Figure 3b–c). Treatment with 0.01 mg/kg ZK 137 316 elevated the mitotic index in this zone as well, but the two higher doses of ZK 137 316 led to a dose-dependent decline in mitotic index despite the dose-dependent increase in the percentage of positive Ki-67 cells (Table I).

In the basalis zone of controls (Figure 3e), there was considerable Ki-67 and mitotic activity, consistent with the mitogenic effects of progesterone on this zone during the luteal phase in macaques (Okulicz *et al.*, 1993). In the basalis, ZK 137 316 treatment caused dose-dependent inhibition of both the Ki-67 (Figure 3f–h) and mitotic indices (Table I) consistent with inhibition of progesterone action.

Endometrial ER and PR

In the functionalis zone of control monkeys, ER staining was very low in the glandular epithelium and almost undetectable in the stroma (Figure 4a), while in the basalis the glandular epithelium retained strong ER staining (Figure 4e). This

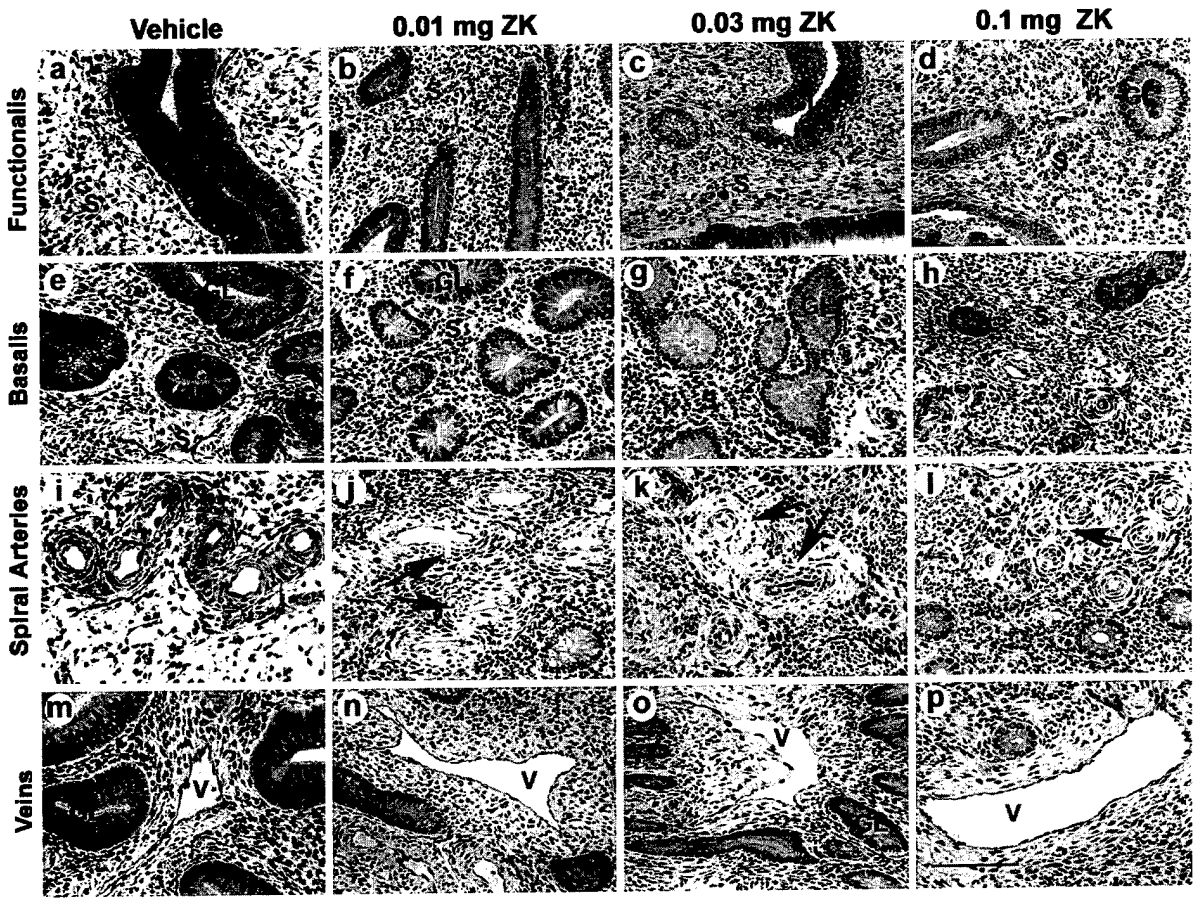

Figure 2. Photomicrographs of GMA embedded, haematoxylin-stained sections of endometrium. (**a–d** functionalis) In controls (**a**) the functionalis was in a differentiated state with an expanded stroma (S) and a columnar secretory epithelium (Gl) with no observable mitotic cells; 0.01 mg/kg ZK 137 316 (**b**) increased compaction of the stroma and reduced differentiation of the glands, which were in a proliferative state; 0.03 mg/kg ZK 137 316 (**c**) resulted in further compacted stroma and more suppressed glands which were almost absent; 0.1 mg/kg ZK 137 316 (**d**), resulted in a very dense stroma and cuboidal glandular epithelium. All figures at the same original magnification (×630). Bar, 100 μm. (**e–h** basalis) In vehicle controls (**e**) the basalis epithelium was in a proliferative state with abundant epithelial mitosis; 0.01 mg/kg ZK 137 316 (**f**) resulted in a slightly compacted stroma, and reduced glandular development, but the glandular epithelium was proliferative; 0.03 mg/kg ZK 137 316 (**g**) resulted in greater suppression of the glands and few mitotic cells were evident; 0.1 mg/kg ZK 137 316 (**h**) resulted in a very compacted stroma and the remaining glandular epithelium was cuboidal and non-proliferative. (**i–l** spiral arteries) In vehicle controls (**i**), normal spiral arteries were evident (arrows indicate arteries). Treatment with 0.01 mg/kg ZK 137 316 (**j**) and 0.03 mg/kg ZK 137 316 resulted in accumulation of extracellular matrix around the arteries. Spiral arteries were more atrophied and rare after treatment with 0.1 mg ZK 137 316 (**l**). (**m–p** endometrial veins) In vehicle controls (**m**), small veins were evident. ZK 137 316 injection at all doses (**n–p**) resulted in degenerative hyalinization and dilation of the veins.

Table I. Uterine wet weight[a] and endometrial morphometrics in vehicle- and ZK 137 316-treated monkeys (n = 3/group; mean ± SE)

	Dose of ZK 137 316			
	Vehicle	0.01 mg/kg	0.03 mg/kg	0.1 mg/kg
Endometrial weight[a] (mg)	510 ± 10[e]	196 ± 111[f]	36 ± 3[f]	43 ± 13[f]
Myometrial weight[a] (mg)	2213 ± 177[e]	2088 ± 1205[e]	1240 ± 185[e]	2406 ± 682[e]
Endometrial/myometrial ratio[b]	0.23 ± 0.02[e]	0.06 ± 0.00[f]	0.04 ± 0.00[g]	0.02 ± 0.00[h]
Functionalis Ki-67 (%)	1.0 ± 0.0[e]	12 ± 3.8[f]	22 ± 2.0[g]	26.0 ± 7.0[g]
Functionalis mitotic index[c]	nd	10.23 ± 3.4[e]	5.21 ± 0.7[f]	1.23 ± 0.4[g]
Basalis Ki-67 (%)	39 ± 4[e]	19 ± 8[e]	3 ± 1[f]	3 ± 2[f]
Basalis mitotic index	4.6 ± 1.1[e]	5.1 ± 2.4[e]	0.5 ± 0.1[f]	0.1 ± 0.1[f]
Stromal compaction[d]	59 ± 3[e]	95 ± 8.7[f]	127 ± 16[f]	116 ± 9[f]

[a]Endometrial and myometrial weights represent measurements from one-half of the uterus.
[b]Endometrial/myometrial weight ratio was calculated to adjust for variation in reproductive tract size.
[c]Mitotic index represents the number of mitotic cells/1000 cells counted.
[d]Stromal compaction represents the number of stromal nuclei/10 000 μm^2 at ×400 magnification.
[e, f, g, h]Means within each row with different superscripts are statistically different.

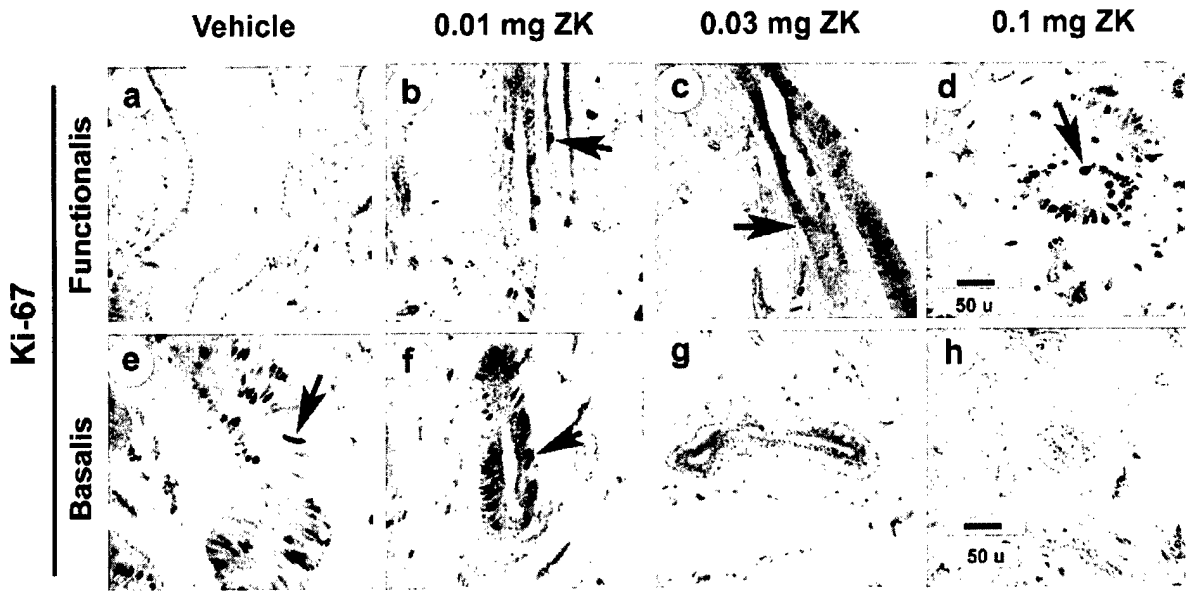

Figure 3. Photomicrographs of endometrium immunostained for Ki-67 antigen. (**a–d**; functionalis) In vehicle-injected controls (**a**) Ki-67 positive cells are almost absent in the glandular epithelium. Treatment with ZK 137 316 at all doses resulted in an increase in the abundance of Ki-67 positive epithelial cells. (**e–h** basalis) Ki-67 positive cells are numerous in both the glands and stroma of controls (**e**). Treatment with ZK 137 316 (**f–h**) caused a decrease in the abundance of Ki-67 positive cells in the basalis glands. Arrows indicate positive nuclei. Bar, 50 μm.

confirms many reports that progesterone suppresses ER in the glands and stroma of the functionalis but not in the basalis during the luteal phase in primates (Brenner *et al.*, 1990; Carson-Jurica *et al.*, 1990; Hild-Petito *et al.*, 1992; Okulicz *et al.*, 1993). Treatment with ZK 137 316 blocked progesterone suppression of ER in the glandular epithelium of the functionalis and increased ER staining in the stroma of the functionalis and basalis in a dose-dependent manner (compare Figure 4b–d).

Progesterone receptor staining was almost undetectable in functionalis glands of controls, as expected from previous reports (Brenner *et al.*, 1990; Hild-Petito *et al.*, 1992; Okulicz *et al.*, 1993) of progesterone suppression of PR in primate endometrium (Figure 4i), but present throughout the stroma and in the glands of the basalis. Treatment with ZK 137 316 at all doses increased PR staining in glands and stroma throughout the entire endometrium. The increase in stromal PR appeared to be dose-dependent (compare Figures 4n–p).

Oviductal morphology

In vehicle-treated controls, the epithelium of the oviductal fimbriae and ampulla was deciliated and non-secretory, consistent with previous reports (Brenner and Slayden, 1994) of progesterone suppression of oestradiol action on oviductal differentiation (Figure 5a). Compared with controls, treatment with 0.01 mg/kg had no obvious effect on oviductal ciliation or secretion, which indicated that progesterone action was not blocked at this dose (Figure 5b). However, the 0.03 and 0.1 mg/kg doses resulted in oviducts that were fully differentiated (Figure 5c and d) with a significant increase in the percentage of ciliation. Only the two higher doses were able to block completely the antagonistic effects of progesterone on oestradiol-dependent oviductal differentiation. Treatment with ZK 137 316 had no significant effect on oviductal mass (Table II).

Discussion

In order to interpret the variety of effects of ZK 137 316 on the reproductive tract in rhesus monkeys, the different actions of oestradiol and progesterone on the oviduct, the different endometrial zones and the spiral arteries should be considered. Steroid actions on the primate reproductive tract have been reviewed elsewhere (Brenner and Slayden, 1994), but a few key points are summarized here. The oviduct and the endometrial functionalis are alike in that oestradiol drives proliferation in both tissues. Over a few days, the stimulatory effects of oestradiol produce enlarged straight glands within a modestly expanded stroma, a so-called 'proliferative' condition in the endometrial functionalis. In the oviduct, oestradiol further stimulates differentiation of the ciliated and secretory epithelium. However, the endometrial basalis in macaques is not responsive to oestradiol, and this zone atrophies and shrinks during the follicular phase. When progesterone rises after ovulation, a variety of progesterone-dependent events occur including antagonism of oestradiol action through the suppression of ER and PR (Brenner and Slayden, 1994). For example, most oestradiol-dependent events in the oviduct and endometrial functionalis are inhibited. Oviductal ciliated and secretory cells atrophy and cellular mitosis in the functionalis is blocked, but progesterone stimulates mitosis in the basalis. Progesterone also has differentiative effects in the endometrium. For example elevated progesterone causes the glands of the functionalis to become saccular and secretory, while the glands of the basalis proliferate, hypertrophy and develop a secretory morphology (Okulicz *et al.*, 1993). Progesterone is also mitogenic for the

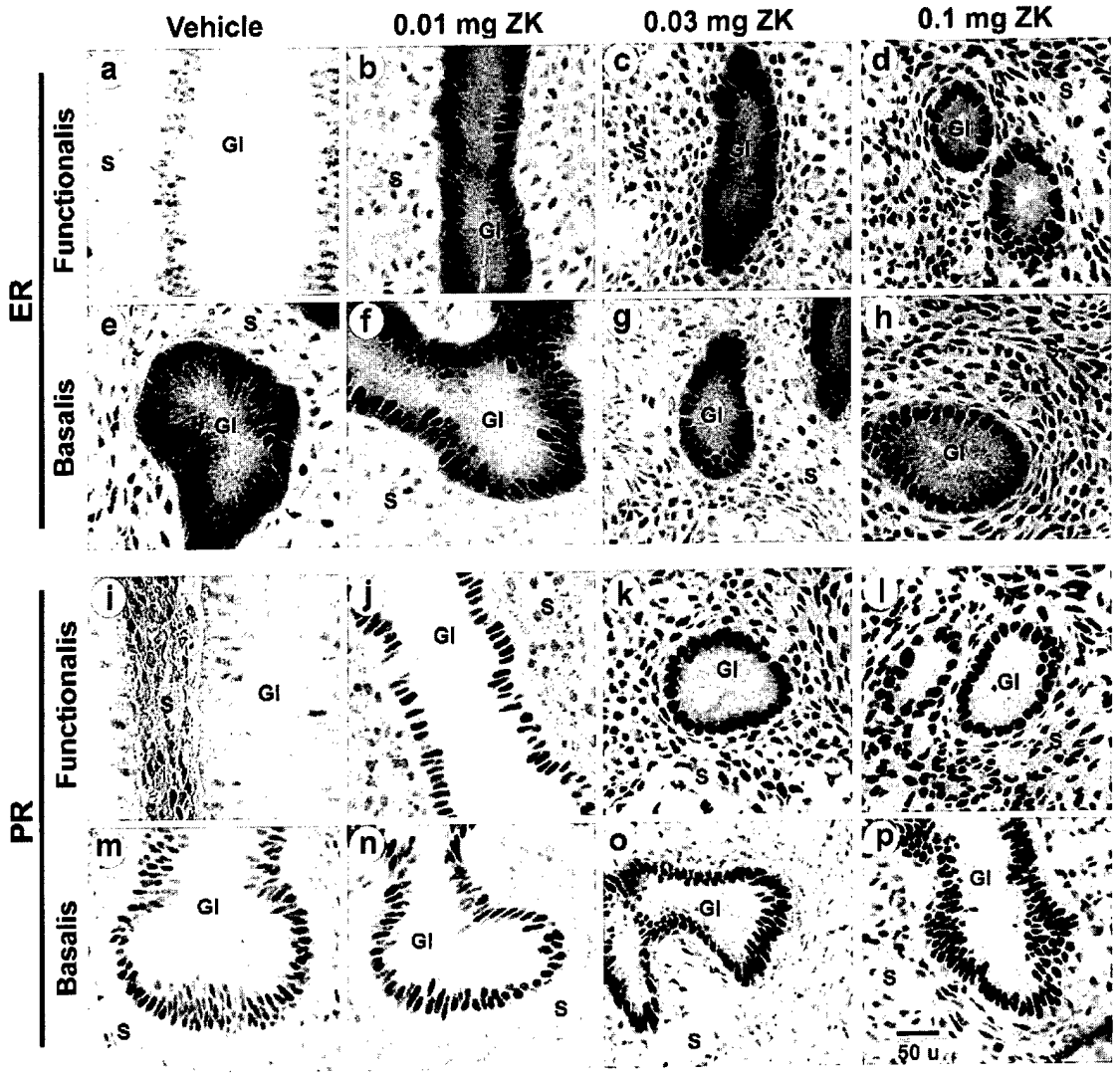

Figure 4. Photomicrographs of endometrium stained for oestrogen receptors (ER) and progesterone receptors (PR) by ICC. (**a–d** functionalis ER) ER was suppressed in the glands (Gl), and almost absent in the stroma (S) of vehicle controls (**a**). ZK 137 316 at all doses (**b–d**) resulted in a striking increase in ER staining in both the functionalis glands and stroma. (**e–h** basalis ER) ER staining was strong in the glandular epithelium of the vehicle and ZK 137 316-treated monkeys. ER was detectable in the stroma of all groups, but the intensity of ER staining was noticeably greater in the 0.1 mg/kg group (**h**). (**i–l** functionalis PR) In vehicle controls (**i**), PR staining was faint in the epithelium, but present in the stroma. ZK 137 316 at all doses increased PR staining in the glands. ZK 137 316 treatment resulted in an increase in stromal PR staining, which was greatest in the 0.1 mg/kg group. (**m–p** basalis PR) In vehicle controls (**m**), staining for PR was strong in the glandular epithelium, but weak in the stroma. Staining in the epithelium remained strong in each of the three ZK 137 316-treated groups. ZK 137 316 resulted in a dose-dependent increase in stromal staining for PR.

spiral arteries, which grow extensively during the luteal phase (Bartelmez, 1957), and it stimulates stromal cell hypertrophy throughout the endometrium (Brenner and Slayden, 1994).

If an antiprogestin were simply to block progesterone action, one would expect to see a dose-dependent blockade of progesterone-dependent events and a concomitant dose-dependent increase in those oestradiol-dependent events suppressed by progesterone. Some of our observations fit this expectation, but some do not. For instance, in the oviduct of ZK 137 316-treated monkeys, we observed a clear dose-dependent increase in oestradiol-dependent ciliation and secretory activity consistent with the suppression of progesterone action. This also supports our previous report on RU 486 (Slayden and Brenner, 1994) that antiprogestins block progesterone but not oestradiol action on the oviduct. Further, in the endometrial basalis, there was a clear dose-dependent inhibition by ZK 137 316 of Ki-67 labelling and mitosis, which is consistent with suppression of the mitogenic effects of progesterone specific to this zone. In the group treated with the highest dose of ZK 137 316, serum progesterone concentrations were very low and oestradiol concentrations were within the physiological range for the follicular phase (Zelinski-Wooten *et al.*, 1997a). This hormonal state by itself should result in lower progesterone-dependent proliferation in the basalis and increased oestradiol-dependent differentiation in the oviduct. Both these effects occurred as expected, uninfluenced by ZK 137 316 treatment. This hormonal state would also be expected to increase cell proliferation in the endometrial functionalis.

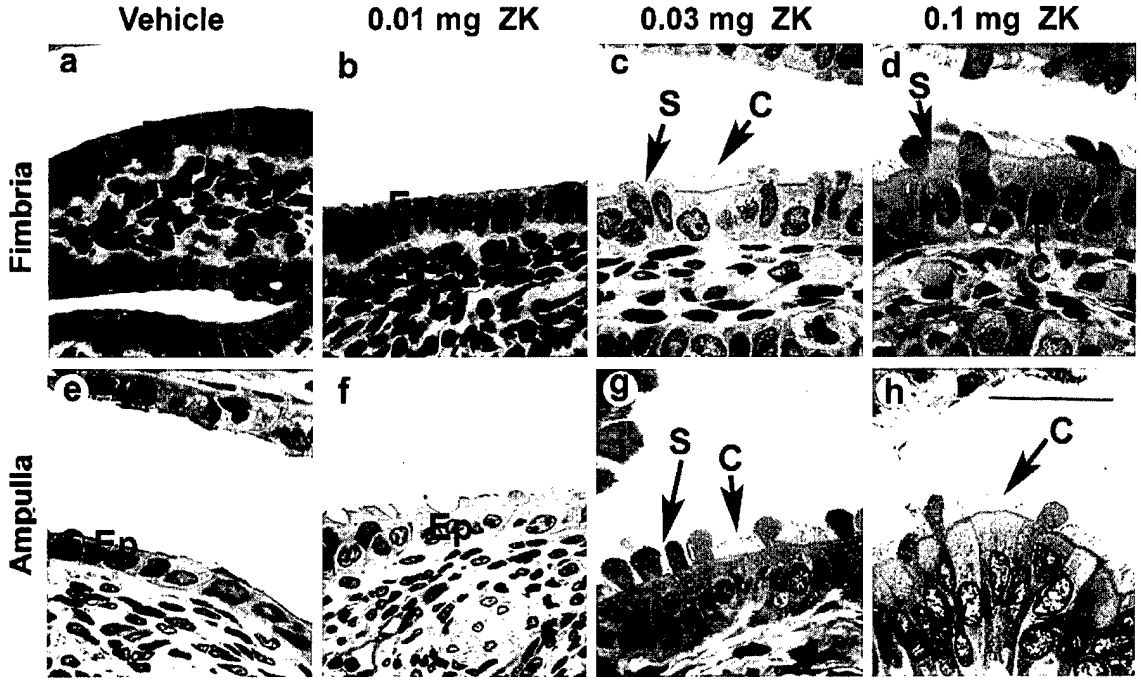

Figure 5. Photomicrographs of haematoxylin-stained GMA sections of oviductal fimbriae and ampulla. In vehicle controls, the fimbriae (**a–d**) and ampulla (**e–h**) were in a non-differentiated state, typical of the effects of mid-luteal phase progesterone. After treatment with 0.01 mg/kg ZK 137 316, the fimbriae (**b**) and ampulla (**f**) were in a non-differentiated state, suggesting that this low dose of ZK 137 316 did not fully block the inhibitory effects of progesterone. Ep indicates epithelium. Injection with 0.03 and 0.1 mg/kg ZK 137 316 resulted in differentiation of the fimbriae (**c** and **d**) and ampulla (**g** and **h**) into a fully ciliated and secretory state. S = secretory cells; C = ciliated cells. Bar, 30 μm.

Table II. Oviductal weights and morphometrics in monkeys following 5 months of treatment with vehicle, 0.01, 0.03 or 0.1 mg ZK 137 316/kg (n = 3/group; mean ± SE)

	Dose of ZK 137 316			
	Vehicle	0.01 mg/kg	0.03 mg/kg	0.1 mg/kg
Oviductal weight (mg)	423 ± 43[a]	333 ± 21[a]	343 ± 110[a]	467 ± 87[a]
Fimbrial ciliation (%)	6.7 ± 2.8[a]	6.0 ± 4.5[a]	34.0 ± 3.5[b]	45.3 ± 3.8[b]
Ampulla ciliation (%)	26.0 ± 3.0[a]	32.0 ± 6.0[a]	32.0 ± 7.0[a]	57.9 ± 5.0[b]

[a,b]Means within each row with different superscripts are statistically different (n = 3).

However, our results indicate that at the highest dose of ZK 137 316, oestradiol failed to drive mitotic activity in the functionalis.

In the endometrial functionalis, there was a ZK 137 316 dose-dependent increase in the Ki-67 index, but only the lowest ZK 137 316 dose led to a concomitant increase in mitotic index. As stated above, the higher ZK 137 316 doses resulted in a dose-dependent decrease in mitotic index. This latter effect is not consistent with a simple dose-dependent inhibition of progesterone action. As noted, at the highest dose, progesterone concentrations were extremely low but oestradiol was within normal concentrations. Therefore in this group the severely atrophied endometrium was partly due to the absence of progesterone and partly due to inhibition of the effects of oestradiol. Because ER and PR staining were intense in all endometrial cells, these data suggest that ZK 137 316 specifically inhibited oestradiol-dependent mitosis in the endometrial functionalis by some mechanism beyond the ER concentration. These data support the report by Cameron *et al.* (1996) that antiprogestins may permit cells to enter the cell cycle under oestradiol influence but prevent them from leaving the cycle through mitosis. Oestradiol also causes some modest growth of the spiral arteries and the stroma during the natural follicular phase (Bartelmez, 1956), and these effects of oestradiol were also inhibited following chronic treatment with the highest dose tested.

The inhibitory actions of ZK 137 316 on the endometrial functionalis complement the growing number of reports indicating that antiprogestins can inhibit some effects of oestrogen (Baron *et al.*, 1987; Wolf *et al.*, 1989; Chwalisz *et al.*, 1991; Slayden *et al.*, 1993). The 'anti-oestrogenic' effects of RU 486 have been called non-competitive because RU 486 does not bind to ER (Neulen *et al.*, 1996). However, these effects are more appropriately referred to as endometrial antiproliferative effects, as they appear to be restricted to this tissue (Slayden and Brenner, 1994). None of the effects of oestradiol on the oviduct appear to be blocked by antiprogestins. The concentrations of endometrial ER and PR, both of which are

oestradiol-dependent proteins, were substantially elevated after chronic ZK 137 316 treatment, as reported for other antiprogestins in other tissues and species (Neulen *et al.*, 1990, 1996; Chwalisz *et al.*, 1991; Slayden *et al.*, 1993; Murphy *et al.*, 1995). It was recently suggested that abnormally high ER in itself might play some role in the inhibition of oestradiol action in the endometrium (Neulen *et al.*, 1996). This possibility should be further explored, but ER are also elevated in the oviduct by RU 486 treatment (Slayden *et al.*, 1993) and oestradiol action is not inhibited in that tissue. The explanation for the restriction of the anti-oestradiol effects of antiprogestins to the macaque endometrium is not known.

In this study, long-term treatment with ZK 137 316 also resulted in degenerative effects on the endometrial arteries and veins. The spiral arteries are unique to the primate endometrium and a blockade of their growth may specifically reduce blood flow. Decreased blood flow was observed in women treated with RU 486 (Reinsch *et al.*, 1994). Reduced vascular support might ultimately reduce the nutritional and oxygenated state of the endometrium, while other regions of the tract such as the oviduct remain unaffected. Collectively, these effects may create a nutritionally deprived endometrium that cannot respond to oestradiol.

The dramatic atrophy of the endometrium produced by ZK 137 316 treatment at low doses which do not inhibit ovarian steroid concentrations, may be due to a combination of several effects. These include: blockade of progesterone action on the spiral arteries, the inhibition of progesterone-dependent basalis growth, the suppression of general stromal function with a specific blockade of progesterone-dependent growth factors such as keratinocyte growth factor (Koji *et al.*, 1994) and insulin-like growth factor II (Giudice *et al.*, 1993), as well as a currently unexplained endometrial specific inhibition of oestradiol action on cell proliferation in the functionalis. Because ZK 137 316 and other antiprogestins have such dramatic antiproliferative effects in the primate endometrium, while the oviducts show no histological abnormalities, it appears that there were no untoward effects of unopposed oestrogen on the endometrium or the oviduct.

Because implantation is unlikely to occur in a severely atrophied endometrium, long-term treatment with low-dose antiprogestin may comprise a novel approach to contraception. The oviductal environment of ZK 137 316-treated animals may also be inimical to normal gamete transport and function, since the oviduct was in a fully differentiated state which was atypical for the luteal phase of the cycle. These oviductal and endometrial effects could act together to potentiate a contraceptive action of ZK 137 316. However, whether there are any deleterious effects of long-term, low-dose antiprogestin therapy on other oestradiol-dependent tissues such as the cervix, the vagina or the mammary glands in non-human primates remains to be established.

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Endometrial effects of progesterone antagonists and novel progesterone receptor modulators (PRMs) in primates

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Progesterone antagonists (PAs; antiprogestins) can modulate estrogenic effects in various estrogen-dependent tissues. These modulatory effects are complex and depend on species, tissue, type of compound, dose and duration of treatment. In non-human primates, PAs, including mifepristone, ZK 137 316 and ZK 230 211, inhibit endometrial proliferation and induce amenorrhea through unknown mechanisms. When administered chronically at relatively low doses these compounds block the mitotic activity of endometrial epithelium and induce stromal compaction in a dose-dependent manner in both spayed and intact monkeys at high estradiol concentrations. These effects were accompanied by an atrophy of spiral arteries. The antiproliferative effects were endometrium-specific, since the estrogenic effects in the oviduct and vagina were not inhibited by PAs. Similar endometrial antiproliferative effects were also found after treatment with the progesterone receptor modulator (PRM, mesoprogestin) J 1042. Importantly, both PAs and PRMs blocked the mitotic activity of the endometrial epithelium in spite of increased expression of estrogen-dependent molecular markers such as ER, PR and KI-67. Our studies indicate that both pure PAs and PRMs selectively inhibit estrogen-dependent endometrial proliferation in the primate endometrium without affecting estrogenic response in other estrogen-dependent tissues nor inducing unscheduled bleeding. The PRMs seem to be more tissue-specific than the PAs in this respect. Our studies indicate that the spiral arteries, which are unique to the primate endometrium, are the primary targets damaged or inhibited by PAs and PRMs. The damage to these unique vessels may underlay the paradoxical, endometrium-specific antiproliferative effects of these compounds. Hence, the properties of PAs and PRMs (mesoprogestins) open up new applications in gynecological therapy and hormone replacement therapy.

ASRM 2000
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ABSTRACTS

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The abstracts will be presented in the ASRM 2000 meeting sessions and are published in the order of their presentation. The abstracts are printed exactly as submitted. Abstracts of special lectures and clinical seminars are not included.

baseline cycle (Day 7 or 14 for biopsies), treatment Cycle 1, and treatment Cycle 3. Cervical mucus scores were classified according to the scoring system developed by Moghissi as a score of 10–15, 5–9, or <5. Endometrial biopsy specimens were classified as proliferative, or mild, moderate, or marked progestational effect. Mean endometrial thickness was determined by ultrasound. Subjects recorded on diary cards when a patch fell off. Safety was evaluated based on adverse events (AEs), vital signs, and body weight.

Results: All 3 groups showed a reduction in the number of cervical mucus scores ≥10 on Days 7 and 14 of Cycles 1 and 3 compared with Day 14 of the baseline cycle (except Triphasil on Day 7, Cycle 3). During Cycle 1, no subject had a score ≥10. During Cycle 3, scores ≥10 were reported for only 2 subjects in the Triphasil group (Day 7) and 1 subject in the EVRA group (Day 14). The majority (75%–100%) of subjects in all 3 groups had a proliferative endometrium at baseline. During Cycle 1, biopsy specimens were predominantly progestational (50%–85%) and, during Cycle 3, all but 1 subject in the Triphasil group (proliferative endometrium) had a progestational endometrium. Endometrial thickness decreased during treatment in all 3 groups. Only 1 patch fell off during the study (at Cycle 1). With the exception of mild application site reactions in 3 subjects in the EVRA group, AE rates were generally similar in the 3 groups and typical of those seen with hormonal contraceptives. There were no serious AEs and no clinically important changes in vital signs. Body weight increased by >5% for 1, 1, and 3 subjects in the EVRA, Triphasil, and Mercilon groups, respectively.

Conclusions: ORTHO EVRA/EVRA is as effective as Triphasil or Mercilon in creating a more sperm-impenetrable mucus and in its effect on endometrial histology and thickness. All regimens were well tolerated.

This study was supported by the R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ

Wednesday, October 25, 2000
2:30 P.M.

O-186

A Contraceptive Patch is Significantly More Effective than Oral Contraceptives (OCs) in Suppressing Follicular Development. [1]R. A. Pierson, [2]D. F. Archer, [3]M. Moreau, [4]M.-C. Audet, [5]M. R. Fluker, [6]C. Bouchard. [1]Royal University Hospital, Canada, [2]Jones Institute for Reproductive Medicine, Norfolk, VA, [3]Centre Hospital de l'Universite de Montreal, Canada, [4]Centre Médical-Halles de Ste-Foy, Canada, [5]4490 Oak St., Canada, [6]Clinique R. S. F. Inc., Canada.

Objectives: Follicular development during transdermal contraceptive and OC use was evaluated in 2 studies. Study A compared a 10-cm^2, 15-cm^2, and 20-cm^2 patch size (20-cm^2 = ORTHO EVRA™/EVRA™ [EVRA™]), which delivers norelgestromin 150 μg and ethinyl estradiol [EE] 20 μg daily to the systemic circulation) and ORTHO-CYCLEN® (oral norgestimate 250 μg + EE 35 μg) after correct dosing and after a 1-day delayed start. Study B compared EVRA, Triphasil® (oral levonorgestrel 50/75/125 μg + EE 30/40/30 μg), and Alesse® (oral levonorgestrel 100 μg + EE 20 μg) after correct dosing and after a 3-day dosing error.

Design: Study A was an open-label (OL), 4-cycle trial involving 32 centers. Healthy females were randomized to a 10-cm^2 patch (n=153), 15-cm^2 patch (n=157), EVRA (n=150), or ORTHO-CYCLEN (n=150). For Cycles 1–3, patch treatment was 3 consecutive 7-day patches (21 days), followed by 1 patch-free week; OC treatment was 1 pill daily for 21 days, followed by 1 pill-free week. Treatments were delayed by 1 day at the start of Cycle 4. Study B was an OL, 5-cycle trial involving 12 centers. Healthy females were randomized to EVRA Group I (n=25), EVRA Group II (n=27), Triphasil (n=22), or Alesse (n=25). Cycles 1–3 and 5 consisted of 21 dosing days (OCs, daily; EVRA, weekly) and 7 drug-free days (correct dosing). A dosing error was planned in Cycle 4, a 10-day cycle in which proper dosing was not followed for Days 8–10. For EVRA Group II, Triphasil, and Alesse, 7 dosing days were followed by 3 drug-free days. EVRA Group I wore 1 patch for 10 consecutive days. Treatment was resumed for all groups on Day 11, the first day of Cycle 5.

Materials and Methods: Maximum mean follicular diameter (MMFD) was determined weekly in a subset of subjects in Study A (n=109) and up to daily in all subjects in Study B by ultrasound measurements. In Study B, between-regimen differences at Cycle 5 were assessed with 2-sided, 95% confidence intervals. Safety was evaluated based on adverse events, vital signs, and body weight.

Results: In Study A (Cycles 1 and 3 combined), MMFDs were 23.4, 23.2, 12.4, and 15.1 mm in the 10-cm^2 patch, 15-cm^2 patch, EVRA (20 cm^2), and OC groups, respectively. Delaying the start of Cycle 4 by 1 day had no effect on MMFD. After correct dosing in Study B (Cycles 1–3), MMFDs were smallest with EVRA (6.8–7.4 mm) vs Triphasil (8.5–12.4 mm) or Alesse (9.8–16.0 mm). After a 3-day dosing error in Study B (ie, at Cycle 5), MMFD was lowest with EVRA: 7.1 mm EVRA Group I, 6.8 mm EVRA Group II, 11.8 mm Triphasil, and 17.1 mm Alesse. At Cycle 5, significant differences were seen between EVRA Group I vs Triphasil (p=0.034) and Alesse (p=0.003), and between EVRA Group II vs Triphasil (p=0.003) and Alesse (p=0.001). All regimens were well tolerated.

Conclusions: After a 3-day dosing error, ORTHO EVRA/EVRA is significantly more effective than Triphasil or Alesse in suppressing follicular development. ORTHO EVRA/EVRA is at least as effective as ORTHO-CYCLEN in suppressing follicular development.

This study was supported by the R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ.

Wednesday, October 25, 2000
2:45 P.M.

O-187

Antiprogestin-Releasing Intrauterine Devices: A Novel Approach to Endometrial Contraception. [1]N. R. Nayak, [1]O. D. Slayden, [2]K. Chwalisz, [3]M. Lehtinen, [1]R. M. Brenner. [1]Division of Reproductive Sciences, Oregon Regional Primate Research Center, Beaverton, OR. [2]Fertility Control and Hormone Therapy Research, Research Laboratories of Schering AG, Berlin Germany. [3]Leiras OY, Finland.

Objectives: Copper containing and progestin-releasing intrauterine devices (IUDs) are highly effective contraceptives, but compliance is sometimes low because of either excessive bleeding or breakthrough bleeding. Because antiprogestins are known to suppress endometrial development and bleeding, an antiprogestin-releasing IUD may be clinically valuable. The main objective of this study was to develop a bleeding free antiprogestin-releasing IUD regimen assuring that the suppression of endometrial growth will have no systemic effect.

Design: We have examined the effects of blank vs antiprogestin-releasing (ZK 230 211, Schering AG, Germany) IUDs in ovariectomized, artificially cycled macaques on bleeding patterns, endometrial structure and proliferation.

Materials and Methods: Six macaques were recruited for this study. Leiras OY, Finland provided antiprogestin-releasing (ZK 230 211) IUDs specially designed for the macaque uterus. Menstrual bleeding pattern, histology of uterus and oviduct, immunohistochemical localization of estrogen receptors, progesterone receptors, and Ki-67 were examined following treatment with blank IUDs (control), high-dose releasing IUDs (26–30 μg/day), and low-dose releasing IUDs (3–4 μg/day).

Results: Acute administration of local antiprogestin by IUD induced bleeding in a progestin primed endometrium, indicating a local blockade of systemic P action, while continued exposure of the endometrium to the antiprogestin releasing IUDs during artificial cycles prevented P-withdrawal bleeding and led to inhibition of endometrial development. An "antiestrogenic" endometrial antiproliferative effect was also observed. Endometrial steroid receptor levels were elevated exactly as after systemic treatment with antiprogestins. In the spiral arteries, proliferation was inhibited and degenerative changes were induced. In control and antiprogestin treated animals, at the end of the artificial luteal phase, the oviductal epithelium was atrophied, non-secretory and deciliated, classic signs of progesterone action in macaques, suggesting there was no systemic action of the antiprogestin.

Conclusions: We concluded that the antiprogestins released by IUDs can act locally to inhibit the effects of both estrogen and progesterone on endometrium, and that antiprogestin-releasing IUDs can be used as a highly effective bleeding free contraceptive strategy.

Supported by Lalor Foundation, Mellon Foundation and UIS DE950242 (Department of Defense).

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and 2.6 kb, for IRF-6. *In situ* hybridization analyses indicated that all three IRFs were expressed at low levels in the endometrial lumenal epithelium (LE) in cyclic ewes, but IRF-2 and IRF-6 were specifically up-regulated in LE and superficial glandular epithelium on Days 15 and 17 of pregnancy. These results suggest that IRF-2 and IRF-6 may be components of an IFNτ-induced signaling cascade which mediates the negative effects of IFNτ on transcription of ERα and perhaps OTR genes in the endometrial epithelium during pregnancy recognition in sheep. Supported by NIH Grant HD32534.

29. OVER-EXPRESSION OF SPERMIDINE/SPERMINE N1-ACETYLTRANSFERASE (SSAT) ALTERS GENOME EXPRESSION PROFILES IN THE REPRODUCTIVE ORGANS OF FEMALE MICE. SH Min, Rosalia CM Simmen, CW Porter, J Janne and Frank A Simmen. Anim. Mol. Cell Biol. Program, University of Florida, Gainesville, FL; Grace Cancer Drug Center, Roswell Park Cancer Institute, Buffalo, NY; University of Kuopio, Kuopio, Finland.

Spermidine/spermine N1-acetyltransferase (SSAT) is the rate limiting catabolic enzyme in polyamine metabolism that, together with polyamine oxidase, re-converts spermidine and spermine ultimately into putrescine. Previous studies have shown that over-expression of SSAT in transgenic mice leads to profound disturbances of polyamine pools in a number of tissues. These alterations are accompanied by dramatic phenotypic changes including ovarian hypo-function and an underdeveloped uterus, resulting in infertility of female transgenic animals. The present study compared the gene expression profiles in uterus and ovary from control (C) and SSAT over-expressing transgenic (T) mice to gain insight into the molecular mechanisms underlying physiological responses to altered polyamine levels. RNAs from uterus and ovaries of C and T groups (n=4 animals) were isolated, pooled and subjected to mRNA differential display RT-PCR (ddRT-PCR). Visual inspection of ddRT-PCR gels suggested that over-expression of SSAT induced major changes in the expression profiles, with altered band intensities observed for ~20% of the ddRT-PCR products between C and T uteri and C and T ovaries, respectively. A subset of candidate cDNA fragments were excised from gels, re-amplified, confirmed for differential expression by Northern Blots, and characterized by sequence analysis. In both tissues, SSAT over-expression induced the mRNAs for lipoprotein lipase (>3-fold) and glyceraldehyde-3-phosphate dehydrogenase (1.5-fold), but decreased mRNA abundance for murine endogenous leukemia virus (4-fold) and endogenous retrovirus (3-fold). In addition, other mRNAs, reported only in the mouse EST database and whose functions are not currently identified, were either up- (n=1) or down-regulated (n=5) in both uterus and ovaries upon SSAT over-expression. These results suggest that polyamines play a critical role in the development of female reproductive organs by altering the expression profiles of a large number of unknown genes. Supported by NIH HD21961 (RCMS, FAS) and NIH NCI CA76428 (CWP, JJ).

30. ANTIPROGESTIN TREATMENT INCREASES ANDROGEN RECEPTOR IN THE MACAQUE REPRODUCTIVE TRACT. Ov D Slayden, Nihar R Nayak, Kristof Chwalisz and Robert M Brenner. Oregon Regional Primate Research Center, Beaverton, OR; Jenapharm GmbH&Co. KG, Jena, Germany.

Antiprogestins (APs) including RU 486 (RU) and ZK 137 316 (ZK; Schering AG), inhibit estradiol (E)-stimulated endometrial growth in primates, but the mechanism of this action is unknown. Androgen treatments have similar E antagonistic effects on the uterus. To determine whether androgen receptors (ARs) play a role in the anti-endometrial effects of APs, we investigated the effect of APs on AR expression in the endometrium and oviduct of E treated, ovariectomized macaques. In experiment 1, animals received either 1) E alone for 28 days (n=6), or 2) E plus 1 mg RU/kg daily (im) for 28 days (n=3). Uterine and oviductal samples were prepared for immunocytochemistry (ICC) with the AR-specific antibody F-39. The remaining tissues were analyzed by radioligand binding and Scatchard analysis with [^{3}H]-R1881. In experiment 2, animals were treated for 28 days with 1) E alone (n=3); 2) E + 0.01 mg ZK (n=4) 3) E + 0.05 mg ZK (n=3); 4) E + 0.1 mg ZK /kg (n=3), and uteri and oviduct were prepared for ICC. The binding assays revealed that AR levels (mean ±SE) in the endometrium and oviduct under E were, respectively, 3.05±0.35 and 4.51±0.49, and under E + RU had increased to 6.37±0.68 and 7.77± 0.36 fmol/μg DNA ($p<0.05$). ICC revealed that after E alone, there was strong AR staining in the endometrial stroma, but the glandular epithelial cells were completely negative. E + RU treatment led to a dramatic increase in epithelial AR staining and some increase in stromal staining. A similar, dramatic, dose-dependent increase in endometrial glandular epithelial AR staining was induced by ZK treatment. In the oviduct, epithelial AR staining was evident after E alone, and AP treatment induced a modest increase in such staining. Because inhibition of E-dependent, epithelial mitotic activity in the endometrial glands is a major effect of APs, and because androgens have similar effects, we speculate that over expression of AR may play a role in the endometrial antiproliferative effects of antiprogestins. Supported by HD-19182, RR-00163 and DAMD17-996-C-6096

SCIENTIFIC PROGRAM & ABSTRACTS

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Scavenger Receptor - Class B (SR-B1) Expression in Endometriosis. Sumathi Ramachandran,*[1] Helen H Hobbs,*[2] Monty Krieger,*[3] Ana A Murphy,[1] Sampath Parthasarathy.[1] *[1]Department of Obstetrics and Gynecology, Emory University School of Medicine, Atlanta, GA, USA; [2]Department of Internal Medicine and Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, TX, USA; [3]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA.*

Aromatase, a key enzyme in the biosynthesis of estradiol has been suggested to be found in endometrial tissue. High density lipoprotein (HDL) provides precursors for steroidogenic tissues and recently a 82 Kd cell surface protein (SR-B1) has been reported to be involved in the binding of HDL to target cells. In the current study, we present evidence suggesting the presence of SR-B1 in endometrial and endometriosis tisssues. Using a polyclonal rabbit antibody raised against the last 14 aminoacids of the mouse SR-B1 we detected two bands (82 and 45 Kd) in the endometrium and endometriosis samples. High levels of the 45 Kd protein after glycanase treatment showed that the low molecular weight protein might be a non-glycosylated form of the receptor. The m-RNA expression of SR-B1 was also found to be increased in the endometriosis tissues. The location of SR-B1 expression in the endometrium supports a role of this receptor in the uptake of high density lipoprotein-cholesterol. Enhanced expression of the scavenger receptor in endometriosis may suggest the fact that this receptor may deliver HDL-cholesterol, and thereby increasing steroidogenesis. It has been reported that high levels of estradiol enhances the severity of endometriosis. Increased levels of SR-B1 in the endometriosis tissue resulted in enhanced levels of estradiol, which is deterimental in the progression of endometriosis. This lends credence to the functional significance of HDL receptor SR-B1 in cholesterol transport and steroidogenesis.

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Endometrial Apoptosis Is Inhibited In Vitro by TNFα in Women with Endometriosis and Is Stimulated by TNFα in Healthy Women. J Ding,* M Shen,* M Gogacz,* DP Braun,* N Rana,* WP Dmowski. *Institute for the Study and Treatment of Endometriosis, Chicago, IL, USA.*

Previous studies from our laboratory demonstrated that spontaneous apoptosis in the uterine endometrium was significantly reduced in women with endometriosis compared with healthy controls and that tumor necrosis factor-alpha (TNFα) stimulated proliferation of uterine endometrial cells in women with endometriosis but inhibited proliferation in women without endometriosis. We hypothesize that TNFα has a differential effect on apoptosis in endometrium of women with and without endometriosis. To test this hypothesis, single cell suspensions of the endometrial cells were prepared from uterine biopsies using enzymatic dissociation for 19 women with and 18 without endometriosis. Endometrial cells were then cultured in medium containing 0 (TNF0), 100 (TNF100) and 200 (TNF200) units/ml recombinant human TNFα. After 24 and 48 hour of culture respectively, cells were harvested and analyzed for apoptosis using the Cell Death Detection ELISA Plus kit (Roche Diagnostics Corporation, Indianapolis, IN). Data were expressed as mean±SD absorbance and analyzed by analysis of variance using SAS statistical analysis system. Results are shown in Table 1. After 24 hr of culture, TNFα marginally stimulated endometrial cell apoptosis for controls (TNF0 vs TNF200, P=0.06); while it did not affect apoptosis for endometriosis (Ps>0.7). After 48 hr of culture, although TNFα treatment did not alter apoptosis level of endometrial cells in control women, it significantly inhibited apoptosis in endometriosis patients (TNF0 vs TNF100, P=0.046; TNF0 vs TNF200, P=0.027). From these results, we conclude that uterine endometrial cells from women with endometriosis react to TNFα abnormally. In women without endometriosis, TNFα induces programmed cell death of endometrial cells; in women with endometriosis, instead of stimulating apoptosis, TNFα may stimulate endometrial cell proliferation. These results further confirm our previous findings.

Table 1. Effect of TNFα on apoptosis of uterine endometrial cells in women with and without endometriosis (mean±S.D. absorbance.

Hour of Culture	24 hr	24 hr	24 hr	48 hr	48 hr	48 hr
TNFα Treatment	TNF0	TNF100	TNF200	TNF0	TNF100	TNF200
Controls	3.01±1.85	3.11±2.13	3.35±2.08	2.90±2.03	2.91±2.05	2.94±2.04
Endometriosis	2.31±1.23	2.36±1.41	2.35±1.50	2.60±1.61	2.32±1.44	2.29±1.56

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Messenger RNA for the FSH Receptor Is Present in Human Endometrium. Anthony M Propst,* Romana A Nowak,* Elizabeth A Stewart. *[1]Department of Obstetrics, Gynecology and Reproductive Biology, Brigham and Womens Hospital - Harvard Medical School, Boston, MA, USA.*

Objectives: The FSH receptor has been previously cloned from human ovarian tissue. The genomic structure of the FSH receptor consists of 10 exons, with exons 1-9 coding for the extracellular domain and exon 10 encoding the transmembrane and C-terminal domains. It has not previouly been identified in endometrium. Our objective was to determine whether messenger RNA for the FSH receptor is present in human endometrium.
Methods : Messenger RNA, extracted from the endometrium of seven premenopausal women, was reverse transcribed into cDNA. The resulting cDNA was amplified by nested PCR for the FSH receptor. Ovarian granulosa cell cDNA was used as a positive control. The first nested primer pair spanned exons 8 through 10. The second primer pair spanned exons 9 and 10, including most of the transmembrane region. These primers spanned introns 8 and 9, which precludes amplification of genomic DNA. PCR products were visualized using Agarose gel electrophoresis and appropriate sized bands were then cloned and sequenced.
Results: RT-PCR produced a 336-base pair fragment in the granulosa cells and in 4 out of 7 endometrial samples. There was 100% homology to the reported sequence for the human FSH receptor for all clones sequenced from granulosa cells and endometrium.
Conclusions: This study demonstrates for the first time the presence of FSH receptor gene expression in human endometrium. Characterization of the full length receptor will be necessary to exclude a truncated or variant receptor.
Supported by grant HD-115301 from the NIH (E.A.S.)

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Vaginal Administration of Antiprogestin ZK 230 211 in Rhesus Macaques. OD Slayden,*[1] K Chwalisz,[2] RM Brenner.*[1] *[1]Division of Reproductive Sciences, Oregon Regional Primate Research Center, Beaverton, OR, USA; [2]Jenapharm GmbH&Co. KG, Jena, Germany.*

Vaginal administration of progesterone (P) is a clinically accepted mode of delivery that produces physiologically effective levels of P in the endometrium, and low levels in the systemic circulation. We hypothesized that this "uterine first pass effect" could also concentrate antiprogestins within the reproductive tract. Here we describe 3 experiments which show that the Schering antiprogestin ZK 230 211 (ZK), delivered via a vaginal gel, can induce P withdrawal bleeding, block P effects on endometrial secretory development, and block estradiol (E_2) effects on endometrial proliferation. **In experiment 1,** ovariectomized macaques were treated sequentially for 14 days with E_2 implants followed by 14 days of E_2+ P to prime the endometrium for menstruation. E_2+P treatment was continued and the animals then received vaginal ZK suspended in 1 ml Replens™ gel for 5 days. Vaginal swabs were performed daily for 15 days to assess menstruation. Four doses of ZK were tested: 2.5 mg (n=2), 5 mg (n=3), 10 mg (n=4) and 20 mg/animal/day (n=1). Two control animals received Replens™ alone, and these animals did not menstruate. Vaginal ZK treatment with doses ≥10 mg/day induced full menstruation beginning on days 6 and 7 after onset of ZK treatment. **In experiment 2,** similar E_2+ P primed animals (n=3) were treated every other day with 10 mg ZK/monkey for 8 days. In this case, all of the animals treated with vaginal ZK menstruated on day 7 or 8 after treatment began. **In experiment 3,** artificially cycled macaques were treated with vaginal ZK (10 mg/day) and either E_2 alone (n=2) or E_2+ P (n=2) for 20 days after which the uteri were prepared for histological analysis. Treatment with E_2 + ZK substantially reduced epithelial cell proliferation and endometrial thickness compared to E_2 alone. Treatment with E_2+ P and vaginal ZK blocked P-induced endometrial secretory differentiation and prevented any increase in epithelial cell proliferation due to unopposed E_2. **In conclusion,** vaginally delivered ZK 230 211 blocked action of systemic P on the endometrium as well as any endometrial proliferative effects of unopposed systemic estrogen. Previous reports indicate that systemically delivered antiprogestins can act as contraceptives, block unwanted uterine bleeding and suppress E_2-dependent endometrial proliferation. Vaginal administration of antiprogestins can induce these same effects without the potential for undesirable systemic effects such as interference with ovarian function.

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College Station, Texas
August 8-11, 1998

Biology of Reproduction **Volume 58, Supplement 1**

365. DOSE RELATED EFFECTS OF THE NEW GENERATION ANTIPROGESTIN ZK 137 316 IN SPAYED AND CYCLING RHESUS MACAQUES. Slayden OD[1], Chwalisz K,[2*] Vidgoff J,[1*] Brenner RM.[1] Div. of Reproductive Sciences, Oregon Regional Primate Research Center, Beaverton OR 97006,[1] Schering AG, Berlin, Germany.[2]

The effects of the Schering antiprogestin ZK 137 316 (ZK) on menstrual cyclicity and ovulation are highly dose dependent (Zelinski-Wooten et al., Hum. Reprod. 13, in press). Here we report two studies of dose dependent effects of ZK in rhesus macaques. In the first study we administered 0.01, 0.03, 0.05, 0.1 or 0.15 mg/kg ZK (IM) for 4 days at the end of an artificial cycle, which was induced by sequential treatment with estradiol (E_2) and progesterone (P) implants in spayed animals. 0.01 mg/kg ZK did not induce menses, 0.03 and 0.05 mg/kg induced minor bleeding detectable only by vaginal swab, and 0.1 and 0.15 mg/kg induced full menses in all animals. This range of doses, from ineffective to fully effective, defines ZK's ability to block P action and induce menses during E_2+P treatment in spayed macaques. In a second study we evaluated two doses, 0.05 and 0.1 mg/kg administered daily (IM) for 40 days to intact, naturally cycling rhesus macaques (N=6/group; mean cycle length 28.9±1.0 days) to evaluate chronic effects on menses and ovarian cyclicity. Both doses blocked frank, overt menstruation in all animals. In the 0.05 mg/kg group, 3 of the 6 monkeys had normal luteal phases and 3 failed to develop a normal luteal phase. Four of the 6 monkeys had normal E_2 surges and 2 did not. Non-surge E_2 levels were typical of the follicular phase in all animals. Similar results were seen in the 0.1 mg/kg ZK group. Animals that had normal luteal function displayed minute bleeding detectable only by vaginal swab at the time of luteolysis. All animals showed frank menstruation 15-20 days after cessation of treatment, had normal-length post-treatment cycles and normal patterns of E_2 and P during the second post-treatment cycle. In summary, intermediate ZK doses will suppress menses in animals that develop normal luteal function, but such doses may also inhibit development of functional corpora lutea. All such effects were reversible. Chronic, low-intermediate dose ZK treatment may be useful in the management of menstrual disorders. Supported by contract DAMD17-96-C-6096.

366. FOURIER HARMONIC ANALYSIS DIFFERENTIATES SPERM NUCLEAR SHAPES AMONG BULLS AND EJACULATES. Ostermeier GC* and Parrish JJ*. Department of Animal Science, University of Wisconsin, Madison, WI.

The objective was to assess the ability of image analysis techniques developed in this lab to differentiate nuclear shapes of sperm surviving cryopreservation both among bulls and ejaculates within bulls. Frozen semen from 3 ejaculates for each of 11 bulls were provided by American Breeders Service. Frozen-thawed sperm were stained with the fluorescent nucleic acid stains YOYO-1 and Hoechst-33342 to identify live and dead sperm. Slides were prepared, examined with epifluorescence microscopy, and 40 digital images of sperm nuclei acquired on each of 2 consecutive days per sample. Fourier harmonic amplitudes 0-5 were computed for 50 live sperm per sample per day. Differences in harmonic amplitude centroids were demonstrated by MANOVA both among and within bulls ($p<0.05$). Despite differences among ejaculates within bulls, larger differences were observed among bulls. To compare mean sperm nuclear shapes among bulls, multiple cluster analyses of the harmonic amplitude centroids were done. Two of the bulls differed in mean sperm nuclear shape from the other 9, and were thus classified as outliers. To examine how sperm nuclear shape variation differed among bulls, the covariance matrix representing the variance of the 6 harmonic amplitudes within a sample on a given day of image analysis, was solved for its trace (TR) and determinate (DET). To allow each harmonic amplitude equal weight, the 6 distributions were first normalized to have a mean of 0 and SD of 1. TR and DET centroids differed among bulls ($p<0.05$). While differences in mean sperm nuclear shape and sperm nuclear shape variation were expected among bulls, differences among ejaculates within bulls were not anticipated since all ejaculates were collected within a 2 week interval. Thus, the ability to identify small differences among ejaculates within bulls demonstrates the sensitivity of these procedures. Future experiments to assess the relationships of sperm nuclear shape and fertility should account for the ejaculate within bull variation. Work supported by the Natl Assoc. of Anim. Breeders and Coll of Ag. and Life Sciences.

367. CONTRACEPTIVE EFFECT OF A DNA VACCINE BASED ON SPERM-SPECIFIC LACTATE DEHYDROGENASE Bo Zhang[1,3], Yuezhao Bao[*2], Jiabao Zhang[*2], Chongwen Duan[*3], Bin Wang[*3], Dayuan Chen[*3] [1]Dept. of Animal and Nutritional Sci., Univ. of New Hampshire, Durham NH 03824; [2]Dept. of Biol., Henan Normal Univ., Xinxiang Henan 453002, CHINA; [3]Institute of Zool., Chinese Academy of Sciences, Beijing 100080, China.

The sperm-specific lactate dehydrogenase C4 (LDH-C4) plays an important role in energy metabolism and physiological function of sperm. This protein has previously been considered as a contraceptive agent. In this study, we investigated whether a DNA vaccine containing Ldh-c, which encodes for LDH-C4, has an antifertility effect. The Ldh-c recombinant DNA vaccine, constructed by inserting Ldh-c cDNA into the eucaryotic expression vector pCMV4, was transfected into HeLa cells and injected (100μg) into mouse leg muscle. Immunoblotting and immunocytochemical assays confirmed the expression of this gene in vitro and in vivo. Furthermore, LDH-C4 antibodies were detected in the inoculated mice sera (n=20). When the antiserum (1:100 diluted) was incubated with normal mouse spermatozoa, sperm capacitation (capacitated sperm/total sperm±SE, n=14) and motility (motile sperm/total sperm×100±SE, n=15) were inhibited. Compared to controls, the capacitation rate was decreased by 25.4±3.2%, while the motility rates at 5, 90 and 120 minutes were decreased by 56.1±1.1% and 5±1.3%, respectively. On the other hand, sperm capacitation and acrosome reaction rates (n=10) were reduced to 7.6±2.1% and 2.8±1.6% in the immunized mice. Moreover, the sperm motility rates (n=11) at 5, 90 and 120 minutes were 55.3±2.3%, 18. 8±1.2% and 4.9±2.3%, respectively. In summary, the Ldh-c DNA vaccine expressed LDH-C4 protein in vitro and in vivo, elicited specific immunological responses, and greatly decreased the sperm quality in the inoculated mice. In conclusion, these results suggested that DNA inoculation is a new, effective and easy-to-use strategy in the development of contraceptive vaccines.

368. ANTIBODIES AGAINST INTER-ALPHA-INHBITOR SUBUNITS INTERFER WITH FERTILITY. Hess KA[1], Chen L[2], and Larsen WJ[2]. Dev Biol[1], Cell Biol, Neurobiol and Anatomy[2], Univ of Cincinnati Med School, Cincinnati, OH.

Some anti-fertility vaccines are designed to interfere with oocyte viability or fertilization through interaction of specific antibody with antigens of the zona pellucida of the oocyte. Successful vaccines have been developed but have been directed against epitopes within the zona pellucida of oocytes of all developmental stages. The production of reversible anti-fertility vaccines is a major goal in current reproductive research. The strategy of the present study is to develop an effective vaccine against epitopes specific to the responding follicle(s) so that oocytes in primary follicles are unaffected. We have found that the serum glycoprotein, inter-alpha-inhibitor (IαI) is only present in responding follicles and that its covalent linkage to newly

News from ASRM 2000...

Embargoed for release
October 24, 2000

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stipton@asrm.org

New Developments in Contraception Reported

San Diego, CA – Two studies discussing new contraceptive methods were presented today at American Society for Reproductive Medicine meeting.

In a multi-center study, researchers in the US and Canada collaborated on a study to examine whether a contraceptive patch would be as effective as oral contraceptives. The investigators compared patches of different size (10, 15 and 20 cm) with oral contraceptives. Ultrasound measurements of follicular development were used to determine the effectiveness of each of the methods. The researchers found that the 20 cm patch was more effective than the smaller ones, more effective than some of the oral contraceptives and as effective as the most effective pill in the study.

An international team led by scientists from the Oregon Regional Primate Research Center is looking to improve an already effective contraceptive method, the Intrauterine Device (IUD). IUD's are known as a very effective method of contraception, however one of the common complications of their use is excessive bleeding. In an effort to reduce the incidence of breakthrough bleeding, the scientists developed IUD's that also released antiprogestin, which reduces uterine bleeding. Special IUD's were developed and used in monkeys. It was found that the antiprogestin releasing IUD's were a very effective bleeding free contraceptive.

"Contraception remains one of the most important areas in reproductive medicine. While there are many good choices available, our patients are always looking for improvements

in contraception. This research paves the way for more and better choices in modern contraception." Said R. Jeffrey Chang, MD, President of ASRM.

ASRM has more than 8,500 members who are devoted to advancing knowledge and expertise in reproductive medicine and biology, including obstetricians-gynecologists, urologists, endocrinologists, research scientists, medical technologists, and allied health professionals. ASRM-affiliate societies include the Society for Reproductive Surgeons, the Society of Reproductive Endocrinology and Infertility, the Society for Male Reproduction and Urology, and the Society for Assisted Reproductive Technology.

Figure 1

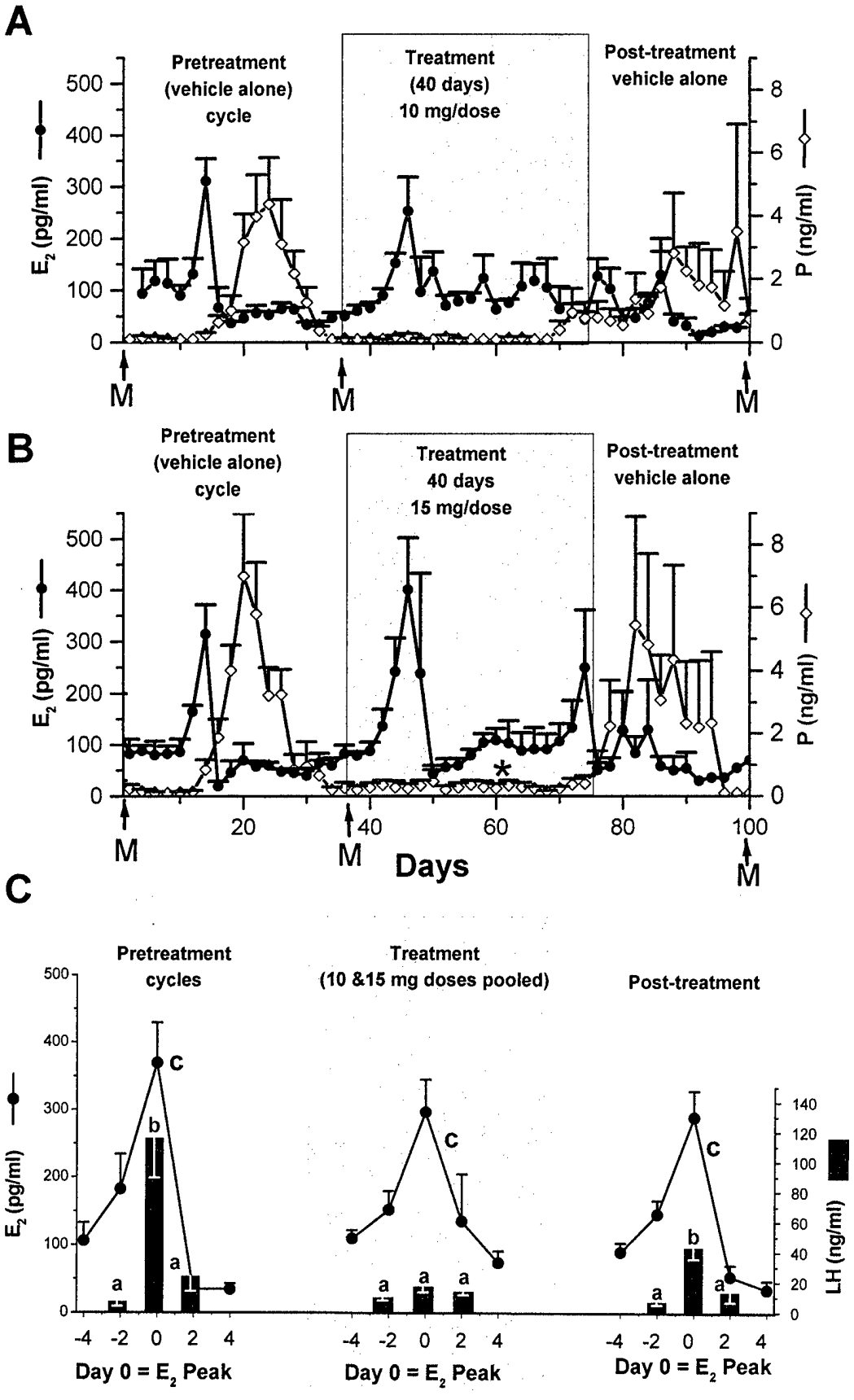
A
Pretreatment
(vehicle alone)
cycle
Treatment
(40 days)
10 mg/dose
Post-treatment
vehicle alone
E2 (pg/ml)
P (ng/ml)
M
M
M
B
Pretreatment
(vehicle alone)
cycle
Treatment
40 days
15 mg/dose
Post-treatment
vehicle alone
E2 (pg/ml)
P (ng/ml)
Days
C
Pretreatment
cycles
Treatment
(10 &15 mg doses pooled)
Post-treatment
E2 (pg/ml)
LH (ng/ml)
Day 0 = E2 Peak
Day 0 = E2 Peak
Day 0 = E2 Peak

Figure 2

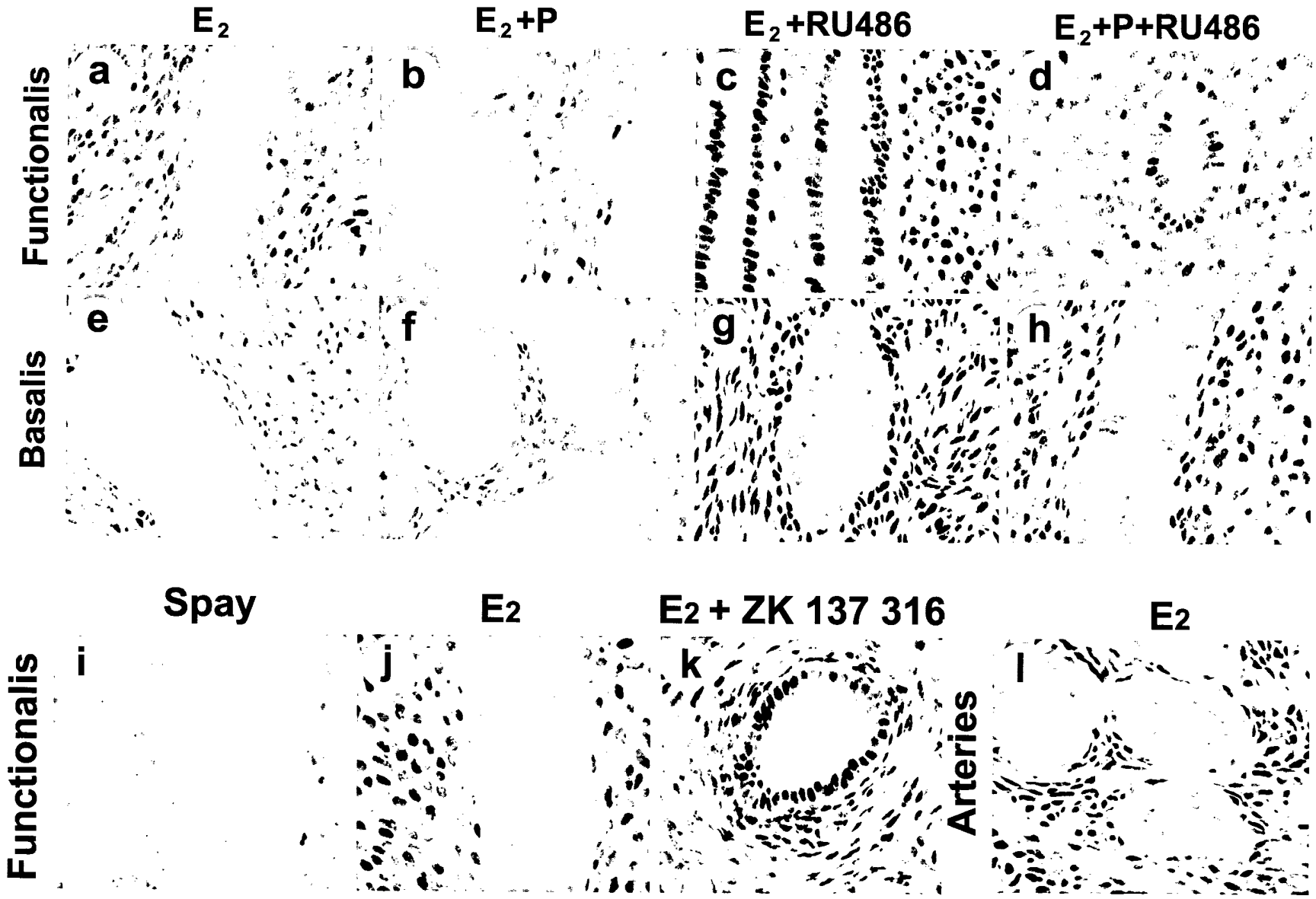
E2
E2+P
E2+RU486
E2+P+RU486
Functionalis
Basalis
a
b
c
d
e
f
g
h
Spay
E2
E2 + ZK 137 316
E2
Functionalis
Arteries
i
j
k
l

Figure 3

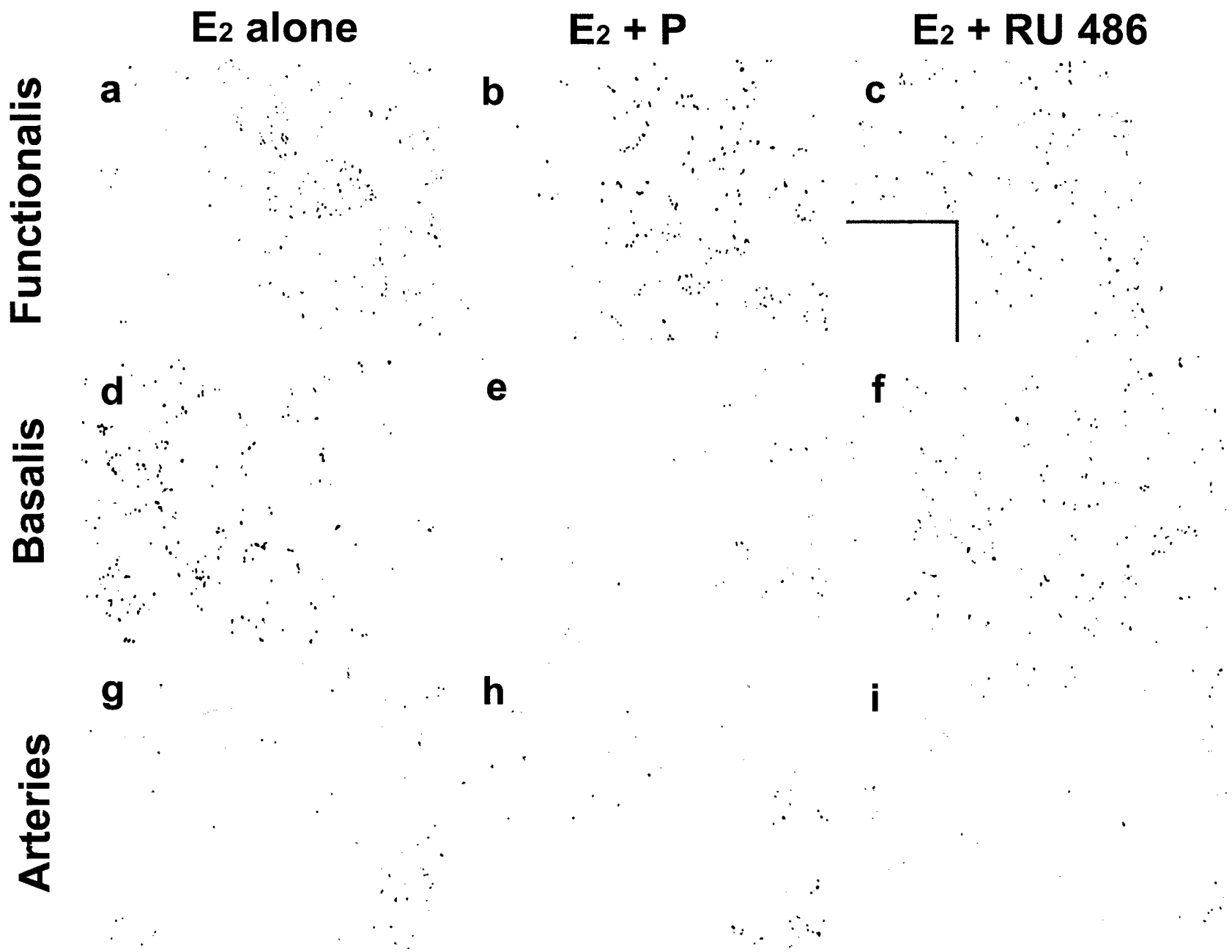
E2 alone
E2 + P
E2 + RU 486
Functionalis
Basalis
Arteries
a
b
c
d
e
f
g
h
i

Figure 4

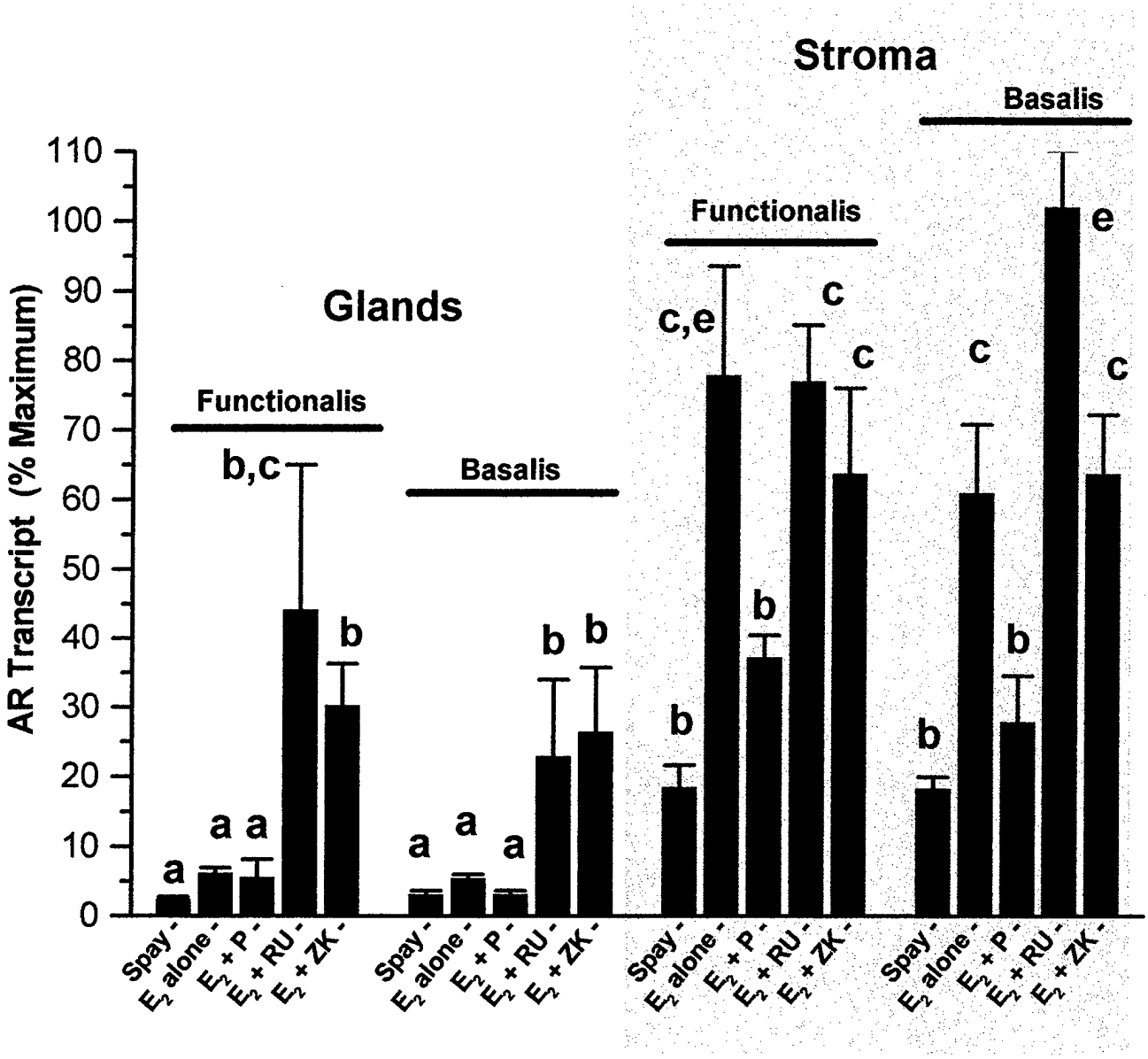
Stroma
Glands
Functionalis
Basalis
Functionalis
Basalis
AR Transcript (% Maximum)
0
10
20
30
40
50
60
70
80
90
100
110
a
a a
b,c
b
a
a a
b b
c,e
b
c
c
b
c
c
e
c
Spay -
E2 alone -
E2 + P -
E2 + RU -
E2 + ZK -

Figure 5